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

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ABSTRACT

Coffee wilt is a vascular disease caused by *Fusarium xylarioides* Steyaert, which is the conidial stage of *Gibberella xylarioides* Hem. and Saccas. CWD can be very destructive to coffee trees (Figure I.II), leading to total loss of investment. It was first reported in 1927 on *Coffea liberica* var. *dewevrei* (formerly *C. liberica* type excelsa, Davis et al., 2006) plantations in the Central African Republic (CAR) (Figueres, 1940). It progressively destroyed this crop during 1930s to 1950s in other Central African countries i.e. Cameroon (Guillemat, 1946; Fraselle, 1950; Saccas, 1951; Muller, 1997). During the same period, it destroyed *C. canephora* in Ivory Coast, causing serious losses and disappearance of some local varieties (Delassus, 1954). In contrast several varieties of *C. canephora* imported from DRC between 1914 and 1933 exhibited some level of field resistance, which was later confirmed by artificial inoculation (Meiffren, 1961). Meiffren (1961) also reported apparent differences for the same materials planted in different areas of the region, i.e. certain *Coffea liberica* and *C. canephora* varieties showing resistance in Ivory Coast were completely susceptible in CAR, suggesting the resistance was either being influenced by environmental conditions or there were different physiological races of the pathogen in different localities of this region. Fraselle (1950) reported CWD attack on *C. canephora* at Yangambi in the DRC in 1948 and subsequently the disease became a serious problem in many parts of the country. In 1957 similar symptoms were reported on *C. arabica* in Ethiopia (Lejeune, 1958).

C. canephora resistant varieties identified in the DRC were used for replanting within the DRC and Ivory Coast (Saccas, 1956; Meiffren, 1961). In Cameroon the disease was eliminated by rigorous systematic uprooting (Muller, 1997) of the *C. dewevri* plantations. These strategies proved to be successful as the disease had declined drastically by the end of the 1950s and it eventually disappeared from Cameroon and Ivory Coast and probably DRC and CAR. The disease remained affecting *C. arabica* in Ethiopia and it is doubtful if any of these strategies were applied. In 1980s a new large scale outbreaks of CWD were reported on *C. canephora* in the north-east of DRC (Flood & Brayford, 1997), from where it spread rapidly to affect this crop in Uganda (1993) and north-west Tanzania (1997).

Chemical control is considered ineffective. Moreever currently, no commercial varieties resistant to the disease are available and it is not recommended to replant on an infected soil.

The main objective of this project was to develop a global strategy to fight the disease, based on durable resistance and adapted to the smallholder's agro systems and to the conditions prevailing in Africa. A multidisciplinary approach was developed to reach this objective with.

Based on the molecular studies and Biological species/Sterility group identified through the mating tests, and specifically in recognition of the sexually incompatible genetic lineages, four species designations have been proposed: 1. *G. abyssiniae* (anamorph, *F. abyssiniae*) sp. nov., responsible for CWD on *C. arabica* in Ethiopia; 2. *G. congoensis* (anamorph, *F. congoensis*) sp. nov., responsible for CWD on *C. canephora* in the Congo basin region (DRC, Uganda and Tanzania); 3. *F. guineensis* sp. nov., described using *C. canephora*-associated strains obtained during the earlier outbreaks in Guinea, Ivory Coast and possibly CAR; 4. *G. xylarioides* (*F. xylarioides*), neotypified using Central African *C. excelsa* strain BBA 62457 (=DSMZ 62457) and strain ATCC 15664 collected during the first CWD epidemic. The first three are previously undescribed species.

Identification of distinct mating types confirmed that *G. xylarioides* is heterothallic and provides an explanation for the common observance of perithecia under field conditions. Given the apparent prevalence of the teleomorph in nature, and assuming that the perithecia so observed are fertile, the limited genetic variability detected within the four species, particularly those responsible for current outbreaks in eastern and central Africa, is somewhat surprising. This may be due to selection pressure imposed by widespread planting of coffee of limited genetic diversity and/or the relative fitness and rapid spread of the few genetic variants identified.

It is apparent from the research that the historical strains are not directly responsible for the current epidemics in East and Central Africa, although their historical role, remains unclear.

In the contemporary infected areas (survey across E. and W. Africa), even conditions of severe disease pressure and when the *C. canephora* and *arabica* species are cultivated in close proximity, CWD has not been observed on *C. arabica* in DRC, Uganda and Tanzania nor on *C. canephora* in Ethiopia.

In opposite the historical strains collected in CAR, Ivory Coast and Guinean are not specific of one *Coffea* species. The hypothesis is once again put forward, that the fungus contemporary population present on *C. canephora* in DRC, Uganda and Tanzania could be derived from a strong foundation effect that counter-selected a specific isolate of the *C. canephora* species. That result clearly shows that the contemporary strains of *Fusarium xylarioides* have an evolutionary potential that

could evolve towards the acquisition of complementary virulence that might prove pathogenic on the other species of *Coffea*.

To fight CWD, different clones (J1/1 and Q3/4) completly field resistant, and more 1000 resistant trees identified by artificial inoculations have been planted in mother garden before field evaluations.

The analysis of *C. canephora* genetic diversity on different wild coffee tree populations from Uganda, form a new genetic group, different from the groups known to date. The genetic diversity of those wild populations reveals a new source of genes likely to be used in future programmes to improve existing commercial varieties, especially for resistance to CWD.

The spatial evolution of the disease analysed with geostatistical tools, indicate the beginning of an epidemic in foci. The size of the foci increases, and they merge to form a continuous set of diseased coffee trees interspersed with groups of resistant coffee trees. From the diseased trees, the spread can be in all directions, infecting neighbouring trees up to a distance of around 10 m. In order to effectively control the disease, especially when up rooting, it is necessary to up root the nearest neighbours of the infected tree, in a radius of at least ten metres. This up rooting is economically feasible if the attack rate in the plot is under 10%

Faced with the threat of CWD, and the discovery of diseased wild coffee trees, effective conservation of those resources "*in situ*" is not guaranteed and should be completed by placing them in an "*ex situ*" conservation collection in different national or international conservation centres.

The findings of this work are of fundamental importance to the future management of CWD and in safeguarding the livelihoods of millions of smallholder coffee farmers across the African continent

SUMMARY OF FINAL REPORT

WP1 PATOGEN DIVERSITY

WP1 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.

Strains were obtained from plant material affected by CWD in Uganda and the Democratic Republic of Congo (DRC). Although the project research focused on the current outbreaks on *C. canephora* in DRC and Uganda it was considered important, for comparative purposes and to gain a fuller understanding of the pathogen, to include strains from *C. arabica* in Ethiopia and *C. canephora* in Tanzania, where the disease is also present. A small number of strains originally isolated from coffee during the earlier and devastating outbreaks in Central and West Africa were also obtained (herein referred to as 'historical strains'). The collections were supplemented by strains acquired from internationally recognised culture collections, including those at CBS, ATCC, DSMZ, CABI E-UK, FGSC[1] and the Museum d'histoire Naturelle de Paris. At CIRAD, CABI and UCL alone, more than 600 strains are now held. The purity of all strains received was assured by sub-culturing from single conidia or ascospores.

WP1 Task 2: (CABI) Identification, storage, and exchange of isolates

The majority of new isolations from plant material were successfully identified to species level and found to be *G. xylarioides*,. Each partner organisation selected a representative group of strains for study, although a number of these were common to the three European partners as strains could be exchanged. However, and to comply with quarantine restrictions, strains held by European partners could not be transferred to the African partners, nor could strains originating in Africa be shared between African partners. While a number of strains are unique to a particular collection, many are therefore held by more than one partner.

Those strains studied and considered to have unique or representative characteristics were deposited for secure, long-term storage (by lyophilisation or under liquid nitrogen) at CIRAD, CABI and/or UCL where suitable facilities are available. During the course of the project, an electronic *Excel* database was developed by CABI.

WP1 Task 3 (CORI-UNIKIN CIRAD-UCL): Evaluation of the variability in isolate aggressiveness using standard inoculation tests.

To provide information on the pathogenic potential of *G. xylarioides* in support of the various laboratory-based investigations, extensive pathogencity testing was undertaken under glasshouse and screen house conditions by project partners in Europe and Africa respectively. Different artificial inoculation techniques were developed by every partner adapted to the local constraints, (dip root, injection by syringe, stem wounding). Assessment of host response to inoculation enabled representative strains of the pathogen of known aggressiveness (virulence) to be identified and included as 'markers' in subsequent trials undertaken to investigate host-pathogen interactions. The primary aims of this work were to assess host specificity and, in relative terms, aggressiveness of strains to a range of coffee species, varieties and clones and resistance within those hosts. The trials clarified the pathogenic nature of those strains assessed and confirmed the apparent specificity to a single coffee species observed under field conditions in relation to those strains associated with *C. canephora* and C. *arabica* in current outbreaks.[2] Of significance, and in contrast, historical strains exhibited a broader host range and induced CWD symptoms on coffee species other than those from which they were originally isolated. Historical strain DSMZ 62457 (=BBA 62457), for example, isolated from *C. excelsa* in CAR in 1955, exhibited

[1] CBS: Centraalbureau voor Schimmelcultures, Netherlands; ATCC: American Type Culture collection, USA; Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany; FGSC: Fungal Genetics Stock Centre, USA; CABI: CAB International, UK.

[2] Host specificity under natural field conditions has again been apparent from the findings of surveys undertaken across the East and central Africa in 2002-3 where, even under conditions of severe disease pressure and when the species are cultivated in close proximity, CWD has not been observed on *C. arabica* in DRC, Uganda and Tanzania nor on *C. canephora* in Ethiopia.

pathogenicity on *C. excelsa, C. canephora* and *C. arabica* and may represent a historical link to those strains currently affecting one or other of the two coffee species.

WP1 Task 4: (CIRAD, CORI, UNIKIN UCL) Description of the fungal life cycle, asexual and sexual phases

At UCL. the teleomorph form of the fungus, commonly observed on coffee trees under suitable conditions in the field, was successfully generated when two strains of opposing mating type were paired. Pairing strains from diverse origins led to the identification of three biological species (BS1-BS3), within which strains were sexually compatible, as well as a sterility group (SG4) comprising a number of reproductively sterile strains. The latter are sexually incompatible with each other and with strains in each of the BS, while the BS are sexually incompatible with each other. Correlation between the genetic and biological attributes of the pathogen was clear, in that BS1 and BS2 comprised those strains associated with *C. arabica* in Ethiopia and with *C. canephora* in DRC, Uganda and Tanzania, respectively. BS3 comprises two historical strains from Central and West Africa, including DSMZ 62457, associated with *C. excelsa*. The identification of mating type (*MAT*) idiomorphs (*MAT*1-1 and *MAT*1-2) by PCR analysis, confirming the hypothesis that *G. xylarioides sensu lato* is a heterothallic fungus, and will now enable mating types to be identified before confirmatory crosses are performed. Taxonomically, *MAT* sequencing and phylogenetic analysis placed the fungus within the African clade of the *Gibberella fujikuroi* species complex (GFC), an important pathogenic fusarium complex, and resolved four distinct phylogenetic species that correspond to the BS and SG revealed in the mating tests.

The various approaches applied by the project partners proved effective in clarifying the underlying variability that exists within the CWD pathogen. Based on the molecular studies and BS/SG identified through the mating mating tests, and specifically in recognition of the sexually incompatible genetic lineages, four species designations have been proposed: 1. *G. abyssiniae* (anamorph, *F. abyssiniae*) sp. nov., responsible for CWD on *C. arabica* in Ethiopia; 2. *G. congoensis* (anamorph, *F. congoensis*) sp. nov., responsible for CWD on *C. canephora* in the Congo bassin region (DRC, Uganda and Tanzania); 3. *F. guineensis* sp. nov., described using *C. canephora*-associated strains obtained during the earlier outbreaks in Guinea, Côte d'Ivoire and possibly CAR; 4. *G. xylarioides* (*F. xylarioides*), neotypified using Central African *C. excelsa* strain DSMZ 62457 and strain ATCC 15664 collected during the first CWD epidemic. The first three are previously undescribed species.

WP1 Task 5 (CIRAD-CABI-UCL): Evaluation of genetic diversity using a range of techniques including PCR and microsatellite markers

A range of fundamentally distinct molecular approaches was applied principally by CIRAD, CABI, UCL and UoK to investigate underlying genetic variability within the CWD pathogen.

The findings show two major groups. The first comprised *G. xylarioides* strains isolated from *C. arabica* affected by CWD in Ethiopia, the second *G. xylarioides* strains isolated from *C. canephora* affected in Uganda, DRC and Tanzania *G. xylarioides* (this group also included one strain from *C. excelsa* in Uganda). No variability was detected within the two groups. As such, it was not possible to precisely define the geographical origin of strains (DRC, Uganda or Tanzania) or in terms of their coffee host. In contrast, greater variability was found between these two groups and the 'historical strains'. Indeed the latter, obtained from *C. canephora* and *C. excelsa* in Central Africa Republic (CAR), Guinea and Ivory Coast in the 1950s and 1960s. Given the limited number of strains involved, uncertainty remains as to how and why the variability observed within the historical strains may have arisen, how it relates to field behaviour and what the relationship is between these strains and those currently affecting *C. canephora*/*C. excelsa* and *C. arabica*.

WP1 Task 6 Synthesis of the results

Concluding points

- *G. xylarioides*, entails in four distinct biological species/sterility groups and constitute the *Gibberella fusarium complex*.
- Taxonomically, these species could be placed within the *Gibberella fujikuroi* species complex.
- Identification of distinct mating types confirmed that *G. xylarioides* is heterothallic and enabled sexual incompatibility to be shown between the four species. It also provides an explanation for the common observance of perithecia under field conditions.

- Given the apparent prevalence of the teleomorph in nature, and assuming that the perithecia so observed are fertile, the limited genetic variability detected within the four species, particularly those responsible for current outbreaks in East and Central Africa, is somewhat surprising. This may be due to selection pressure imposed by widespread planting of coffee of limited genetic diversity and/or the relative fitness and rapid spread of the few genetic variants identified.
- It is apparent from the research that the historical strains are not directly responsible for the current epidemics in East and Central Africa, although their historical role, if any, remains unclear. Furthermore, it is unclear what role thay had in the earlier widespread and very destructive outbreaks in West and Central where other, as yet unidentified, variants may have been involved.

WP2 – HOST/PATHOGEN INTERACTION

WP2 Task 1 (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.

To provide information on the pathogenic potential of *G. xylarioides* in support of the various laboratory-based investigations, extensive pathogencity testing was underaken under glasshouse and screen house conditions in Europe and Africa. To facilitate this work, various host plant inoculation techniques, including treatment of roots with a spore suspension of the fungus, wounding plantlets with a spore carrying scalpel and inoculation of suspension into the stem, were evaluated and standardised methods developed. Assessment of host response to inoculation enabled representative strains of the pathogen of known aggressiveness (virulence) to be identified and included as 'markers' in subsequent trials undertaken to investigate host-pathogen interactions.

WP2 Task 2 (CORI-UNIKIN) Conduct similar field inoculations in Africa to validate the inoculation method

In Uganda, *C. canephora* clones were assessed for resistance in the field and the results were compared with results of artificial inoculation obtained by different inoculation techniques on clones and open pollinated seedlings in screen house at Kituza and climatic chamber at CIRAD.
Field resistance was confirmed in artificial inoculation performed on rooted cuttings in the screen house at CORI, although disease levels on the clones were not exactly the same. Moreover, the correlation between mortality among clones in the field and in the screen house (rooted cuttings) was significant ($p=0.006$). This indicates that field and screen house assessments give comparable resistance results and both protocols can reliably be used for assessing resistance to CWD in *C. canephora*.

This implies artificial inoculations can be used to reduce time and cost of initial assessment of resistance and are convenient for preselection test.

WP2 Task 3. Analysis of host specificity interaction

To develop a breeding program it is necessary to define host specificity and, in relative terms, aggressiveness of strains to a range of coffee species, varieties and clones and resistance within those hosts. The trials clarified the pathogenic nature of those strains assessed and confirmed the apparent specificity to a single coffee species observed under field conditions in relation to those strains associated with *C. canephora* and C. *arabica* in current outbreaks. Host specificity under natural field conditions has again been apparent from the findings of surveys undertaken across the East and central Africa in 2002-3 where, even under conditions of severe disease pressure and when the species are cultivated in close proximity, CWD has not been observed on *C. arabica* in DRC, Uganda and Tanzania nor on *C. canephora* in Ethiopia. Of significance, and in contrast, historical strains exhibited a broader host range and induced CWD symptoms on coffee species other than those from which they were originally isolated. Historical strain DSMZ 62457, for example, isolated from *C. excelsa* in CAR in 1955, exhibited pathogenicity on *C. excelsa*, *C. canephora* and *C. arabica* and may represent a historical link to those strains currently affecting one or other of the two coffee species.

These results highlights the evolutionary potential *of F. xylarioides*. While the contemporary isolates are specific to a Coffea species, the probability of the contemporary population evolving towards the acquisition of a broader host spectrum cannot be ruled out. This means that the selections currently in progress should be assessed in relation to their specific isolate but also in relation to isolate DSMZ62457.

WP2 Task 4 Combine all these observations to propose a hypothesis on the nature of the resistance

Field resistance of *C. canephora* shows a a range of percentage mortality from 0 to 96%. Disease period (duration from appearance of first symptoms to death of the tree) was also variable between clones, ranging from one to 16 months. Generally resistant genotypes had long disease periods, however certain susceptible genotypes (Q/1/1) had exceptionally long disease periods and

also certain resistant genotypes (R/1/4) had very short disease periods. These observations suggest there is variation in resistance mechanisms among the genotypes, which implies different genes conditions the resistance and in different ways and thus further supporting the hypothesis of resistance to CWD in *C. canephora* being polygenic. But with clones J/1/1 and Q/3/4 completely resistant to this disease in the field, the nature of resistance can be confirmed after analyse of progenies derived from specific crosses of resistant/susceptible genotypes

A comparison of CWD resistance among *C. canephora* genotypes in the field and under artificial inoculations in the screen house and climatic chambers revealed that field, screen house and climatic chamber results are comparable and convenient for pre-selection test. However, differential reaction was observed with clone B2/1 between aerial inoculation by stem wounding and inoculation by root wounding (root dipping). This suggests there are different mechanisms of resistance expressed by the ability of plant to resist penetration of the fungus either through roots or aerial parts. Bugbee and Sappenfiled (1968) reported a similar resistance mechanism for *Fusarium oxysporum* f. sp. *vasinfectum* in Cotton (*Gossypium hirsutum*).

These first results on nature of the resistance are preliminaries and research will be pursuit in further experiment in the frame of a new artificial pollination plan.

WP3 – BREEDING FOR RESISTANCE

WP3 Task 1: Identify sources of resistance through field assessment

Analysis of disease data on *C. canephora* half sib progenies from different populations (wild plants from Itwara and Kibale forest, feral trees from Kalangala islands isolated in Lake Victoria and cultivated trees of Erecta and Nganda sub-groups)revealed that Kalangala and Itwara populations were highly resistant to CWD, highlighting the importance of these populations as sources of resistance genes for inclusion in the commercial varieties. The Nganda and Erecta populations were very susceptible, while Kibale progenies were moderately susceptible. Significant variability was also observed within each of these populations.

C. canephora genotypes in the germplasm collections at CORI and trees surviving among farms devastated by the CWD were artificially tested in the screen house for resistance. Over 1500 resistant individual trees were identified and they have been planted in mother gardens for multiplication and field evaluations (for resistance to diseases, quality and yield) as clones.

Artificial tests performed on seedlings progenies of various *C. arabica* varieties found this species resistant to CWD in Uganda. Similar tests performed on F1 Arabusta (*C. arabica* x *C. canephora*) clones found the Arabusta genotypes resistant to the CWD in Uganda. This confirmed that CWD is not a problem of *C. arabica* and this species together with the F1 Arabusta can be used as sources of resistance genes to control CWD of *C. canephora* in Uganda.

WP3 Task 2: Collect seeds from genotypes representative of the available germplasm, dispatch them for screening.

In order to carry out studies on the genetic diversity of *Coffea canephora* and also assess resistance, it was essential to have individual plants representing the maximum genetic diversity known in this species. To achieve that, different contributors took part in these exchanges and different sources were asked for. First of all, the existing genetic resources were called upon:
- the collections of the two partners, CORI and UNIKIN,
- the NARS in Ivory Coast, which accepted and exchange of planting material and its evaluation
- CIRAD, which used some of the genetic resources preserved in French Guiana
- CATIE (Costa Rica), which supplied seeds of different *Coffea* species for the analysis of interactions.

Then, in order to maximize the genetic diversity studied, surveys were made of unaffected coffee trees in Uganda and DRC in high-pressure zones.
Lastly, surveys were made of coffee trees in the primary forests of Uganda, to enhance our knowledge of the genetic structure of this species.

Berthaud (1986) first identified two diversity groups, a Congolese group, which comprised of genotypes from CAR, Congo basin and Cameroon and a Guinean group, which consisted of genotypes of wild origin from Ivory Coast. Montagnon et al. (1992), proposed a substructure in the Congolese group with two sub-divisions SG1 & SG2. Dussert et al. (2003), placed *C. canephora* genotypes of cultivated and wild origins into five diversity groups, adding two groups B and C to the Congolese group. Recent studies using SSR markers on *C. canephora* among other coffee species (Poncet *et al.* 2004) and on *C. canephora* alone (Cubry et al. 2005) confirmed the structure described by Dussert et al. (2003).

Representative genotypes of wild coffee from primary forests were analyzed using 24 microsatellite markers. The accessions were collected from 5 populations in Kibale forest and 6 populations of Itwara forest and from 4 populations in Kalangala islands forest (feral population). Representative genotypes of the known Guinean and Congolese *canephora* groups were included as controls. Eighteen of these markers were used to compare Ugandan populations with previously known genetic diversity groups of the species. Ugandan *C. canephora* was found to be highly diverse with a genetic sub-structure consisting of wild, feral and cultivated populations. Uganda *C. canephora* was found to be different from previously known diversity groups, implying it forms another diversity group within the species.

WP3 Task 3: Conduct screening tests in both Africa and Europe using isolates with a wide range of aggressiveness.

There are significant genetic differences for resistance between different Ugandan *C. canephora* populations (Kalangala islands, Itwara and Kibale primary forests, Nganda and Erect phenotypes, plant material from hot spots). This suggests that genes for CWD resistance are differently distributed among *C. canephora* populations in Uganda.

At CIRAD, *coffea canephora* accessions from the different genetic groups were evaluated. Coffee trees displaying infected plant rates below 10% were considered in the first analysis to be tolerant and possibly a source of genes of resistance for a breeding programme.
The resistance of the coffee trees belonging to the Guinean group was confirmed.
Moreover, progenies found to have a mortality rate of 0% or under 5% will have to be confirmed and will be selected as a source of resistance to Coffee Wilt.

In DRC, a different level of resistance was observed in plant material from Luki, Kiyaka.

WP4 Task 4: Establish multi-site field trials of wilt resistant/tolerant varieties.

At CORI, among the genotypes studied, clone J/1/1 was completely resistant to CWD in the field and clone Q/3/4 was highly resistant. These clones will have to be re-evaluated for CWD resistance in on-farm multi-site trials under different agro-ecological conditions before they are released to the farming community.
At UNIKIN, first resistant coffee trees identified must be validated also in different agroecological *C. canephora* growing areas within DRC.

WP5 Task 5: Study inheritance of CWD resistance

Analysis of disease data on *C. canephora* half diallel progenies found significant general combining ability for CWD susceptibility/resistance, implying there is an additive genetic relationship between parents and offspring for CWD resistance. Broad sense heritability estimated from the same progenies at a disease level of 55-65% plant mortality was moderate (0.27-0.33) and corresponding narrow sense heritability was low (less than 0.15). Broad sense heritability estimated from an array of clones, within the same disease range, was medium (31-35). This shows that CWD resistance is heritable and therefore progenies of crosses between susceptible and resistant parents are expected to have better resistance than their susceptible parents.

WP6 Task 6 Propose a breeding strategy for durable resistance

Although CWD has taken precedent, yield and quality are very important aspects of the coffee variety improvement programme in Uganda. Hence specific crosses of CWD resistant genotypes with the current commercial clones will be carried out. Progenies will therefore be planted and evaluated in on-station field trials as individual trees for all traits equally and genotypes within acceptable limits will be selected. Selected individuals will be multiplied as clones and planted in multi-site trials for re-assessment for adaptation and adoption. Clones that perform well will be selected for release to farmers as clonal varieties.

Based on field reports from Uganda, DRC, Ethipia and results of previous artificial inoculations *C. arabica* can also be a source of resistance for controlling coffee wilt disease in *C. canephora*.

Bearing in mind the ongoing degradation of natural biodiversity reserves, it is important that the *C. canephora* diversity observed in this study be protected. Because of threats from coffee wilt disease and other unforeseen natural disasters on *C. canephora* genetic resources, efforts will be made to find a collaborator or an international germplasm collection for conservation in multiple conservation centres in different countries to preserve them.

WP4 – DISEASE EPIDEMIOLOGY

WP4 Task 1: Identify sites to characterise climate soil, variety, where the epidemic can be surveyed during the project.

In Uganda, on-farm observations started in 2002 on development and spread of CWD at selected smallholder coffee farms in Mayuge and Iganga in Eastern Uganda and Masaka and Rakai in Central Uganda 2 farms per district and to the field scale on the coffee research station in two experimental trials. In DRC the survey started at the beginning of the project in the different province affected by the wilt.

WP4 Task 2 (CORI, UNIKIN): Description of the spatio-temporal spread of the disease.

Geo-statistics has been used to quantify the degree and range of spatial dependence of variables and to quantitatively characterize changes in the spatial patterns of disease over time

The study shows that at early stages of CWD epidemic, a more or less random distribution pattern was observed. In time, foci of diseased and dead plants were formed, expanding in all directions. The high structural or sample variance derived from semi-variance analysis revealed that the spatial dependence for the disease spread is high, which implies that plant to plant infection plays a major role in CWD spread. However, the actual method(s) by which CWD spreads e.g. through root contact between diseased and healthy trees, by ascospores or conidia; is still unclear. Moreover, the source for initial infections which will start of an epidemic remains a key question to be answered by more in depth studies of the epidemiology of CWD.

The effective range, derived from semi-variance analysis of assessments with host effects indicated that diseased coffee trees can infect healthy coffee trees up to approximately 3 coffee trees (10 m) away. This dependence distance was initially shorter, between one and two coffee trees, but increased with increasing disease incidence.

The effective range calculated from assessment without host effects, of approximately one coffee tree (± 3 m), was shorter and more constant. Together with the result from the kriging analysis, which showed small, albeit measurable displacements of disease foci when comparing analyses with and without host effects, these results also show the importance of the coffee genotype in the spread of coffee wilt disease.

When the disease has invaded a *C. canephora* field, it will continue devastating the coffee trees until all susceptible trees are affected and will thus eventually die.

WP4 Task 3 & 4 (CORI,UNIKIN): Evaluation of the duration of survival form of the pathogen and the ways and duration of survival of the pathogen in the field..

It is apparent from this study that disease spread within the field can occur from one infected plant to the next plant probably through the root contact. Dry wood in the field is the major source of inoculum, which forms abundantly in the form of perithecia. Human activities can accelerate disease spread within the field and beyond. This information can be used to restrict people from moving plant parts and to burn all infected plants after uprooting.

WP4 Task 5: Elaboration of a simplified model of the epidemic and proposition of adapted recommendations.

These observations imply that, for effective control of coffee wilt disease by uprooting infected trees, up to 3 healthy looking trees neighbouring a diseased tree should also be uprooted on first observation of CWD symptoms.

Practical implication of these results is that this intervention is only limited to low disease levels, at probably 5% incidence. At higher disease incidence, diseased trees will be too many and uprooting becomes too costly (In Uganda it costs about US $0.5 to uproot a diseased coffee tree). Secondly, at a disease incidence of more than 5% infection points are many and uprooting 3 trees in all direction of each of these initial points will results into uprooting a large proportion of the crop, leaving the field virtually void of coffee trees. Lack of such information may have contributed to the failure of intervention by uprooting in Uganda (Wetala et al., 2000), where uprooting trees with disease symptoms was emphasized and irrespective of the disease incidence in the field.

CONSOLIDATED SCIENTIFIC REPORT

Objectives

Coffee wilt is a vascular disease caused by *Fusarium xylarioides* Steyaert, which is the conidial stage of *Gibberella xylarioides* Hem. and Saccas. CWD can be very destructive to coffee trees (Figure I.II), leading to total loss of investment. It was first reported in 1927 on *Coffea liberica* var. *dewevrei* (formerly *C. liberica* type excelsa, Davis et al., 2006) plantations in the Central African Republic (CAR) (Figueres, 1940). It progressively destroyed that crop from the 1930s to 1950s in other central African countries, e.g. Cameroon (Guillemat, 1946; Fraselle, 1950; Saccas, 1951; Muller, 1997). During the same period, it destroyed *C. canephora* in Ivory Coast, causing serious losses and the disappearance of some local varieties (Delassus, 1954). In contrast, several varieties of *C. canephora* imported from DRC between 1914 and 1933 exhibited some level of field resistance, which was later confirmed by artificial inoculation (Meiffren, 1961). Meiffren (1961) also reported apparent differences for the same materials planted in different areas of the region, i.e. certain *Coffea liberica* and *C. canephora* varieties showing resistance in Ivory Coast were completely susceptible in CAR, suggesting the resistance was either being influenced by environmental conditions or there were different physiological races of the pathogen in different localities of that region. Fraselle (1950) reported CWD attacks on *C. canephora* at Yangambi in DRC in 1948 and subsequently the disease became a serious problem in many parts of the country. In 1957 similar symptoms were reported on *C. arabica* in Ethiopia (Lejeune, 1958).

Resistant *C. canephora* varieties identified in DRC were used for replanting within DRC and Ivory Coast (Saccas, 1956; Meiffren, 1961). In Cameroon the disease was eliminated by rigorous systematic uprooting (Muller, 1997) of the *C. dewevri* plantations. These strategies proved to be successful as the disease had declined drastically by the end of the 1950s and it eventually disappeared from Cameroon and Ivory Coast and probably DRC and CAR. The disease continued affecting *C. arabica* in Ethiopia and it is doubtful if any of these strategies were applied. In the 1980s new large-scale outbreaks of CWD were reported on *C. canephora* in the north-east of DRC (Flood & Brayford, 1997), from where it spread rapidly to affect that crop in Uganda (1993) and north-west Tanzania (1997).

Chemical control is considered ineffective (Muller, 1997). Moreover, no commercial varieties resistant to the disease are currently available and replanting on an infected soil is not recommended.

The main objective of this project was to develop a global strategy to fight the disease, based on durable resistance and adapted to smallholder agrosystems and to the conditions prevailing in Africa. A multidisciplinary approach was developed to reach this objective with a emphasis on:

Genetic diversity of *Fusarium xylarioides*

- To characterise the population structure in the different infected areas
- To characterise the sexual cycle
- To define the *Fusarium* sp. responsible for the disease

Characterisation of host/pathogen interaction

- To develop an artificial inoculation method
- To define the nature of the host/pathogen interaction
- To characterise the aggressiveness of *F. xylarioides*

Breeding strategy

- To collect new germplasm
- To characterise the genetic diversity of local germplasm
- To identify resistant varieties
- To analyse the inheritance of resistance

Epidemiology

To define the factors influencing the development of epidemics, in relation with the agro-ecological environment.

Results achieved
The results achieved are presented by WP

WP1 – Pathogen diversity

To date, efforts to manage CWD effectively have been hindered by our limited knowledge of the pathogen, *G. xylarioides*, and how it behaves. Specifically, it is important to understand to what extent variability exists within the pathogen population from a morphological, genetic, biological and pathogenic perspective to enable us to know precisely what we are dealing with and to support the development and introduction of a suitable management strategy. This is particularly important where quarantine measures and the use of host resistance are considered to be major and long-term components of the strategy, as is the case with CWD. During the course of this project a range of scientific approaches were employed by the various project partners in Africa and Europe to assess, as part of Work Package 1, the extent of variability that exists within the CWD pathogen. For the most part these proved very effective in providing new knowledge of relevance to the disease and its future management. The following is a summary, or synthesis, of the findings and conclusions of that work. Further details of specific components are available in other sections of the Final Project Report, including the individual partner reports.

In order to adequately investigate variability, it was necessary to firstly acquire a comprehensive range of *G. xylarioides* strains for study that represented the known spatial and temporal occurrence of the disease. Fungal collections were therefore established by the five project partners, CABI, CIRAD, UCL, CORI and UoK[1], at their respective institutes.

WP1 Task 1 (CORI-UNIKIN with the 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.

Strains were obtained principally by collection of plant material (namely wood pieces) affected by CWD in Uganda and the Democratic Republic of Congo (DRC), where the disease is currently present; and isolation and purification of *G. xylarioides* and associated *Fusaria* from that material. Although the project research focused on the current outbreaks on *C. canephora* in DRC and Uganda it was considered important, for comparative purposes and to gain a fuller understanding of the pathogen, to include strains from *C. arabica* in Ethiopia and *C. canephora* in Tanzania, where the disease is also present. A small number of strains originally isolated from coffee during the earlier and devastating outbreaks in central and western Africa were also obtained (herein referred to as 'historical strains'). In these regions, and through widespread uprooting and destruction of affected plantations and replanting with resistant coffee in the mid 1900s, the disease was considered to have been successfully eradicated. The collections were supplemented by strains acquired from internationally recognised culture collections, including those at CBS, ATCC, DSMZ, CABI E-UK, FGSC[2] and the Museum d'histoire Naturelle de Paris. At CIRAD, CABI and UCL alone, more than 600 strains are now held. The purity of all strains received was assured by sub-culturing from single conidia or ascospores.

WP1 Task 2: (CABI) Identification, storage, and exchange of isolates

The majority of new isolations from plant material were successfully identified to species level and found to be *G. xylarioides*, although a number of other *Fusaria*, including *F. stilboides*, *F. lateritium*, *F. solani* and *F. oxysporum*, were also obtained, sometimes in association with *G. xylarioides*. Several of the *G. xylarioides* strains were derived from mono-ascospores produced through mating tests undertaken at UCL. Each partner organisation selected a representative group of strains for study, although a number of these were common to the three European partners as strains could be exchanged. However, and to comply with quarantine restrictions, strains held by European

[1] CABI: CAB International, UK. CIRAD: Centre de Cooperation Internationale en Recherche Agronomique pour Le Developpement, France. UCL: Université Catholique de Louvain, Belgium: CORI: Coffee Research Institute, Uganda: UoK: University of Kinshasa, Democratic Republic of Congo.

[2] CBS: Centraalbureau voor Schimmelcultures, Netherlands; ATCC: American Type Culture collection, USA; Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany; FGSC: Fungal Genetics Stock Centre, USA; CABI: CAB International, UK.

partners could not be transferred to the African partners, nor could strains originating in Africa be shared between African partners. While a number of strains are unique to a particular collection, many are therefore held by more than one partner.

Those strains studied and considered to have unique or representative characteristics were deposited for secure, long-term storage (by lyophilisation or under liquid nitrogen) at CIRAD, CABI and/or UCL where suitable facilities are available. During the course of the project, an electronic *Excel* database was developed by CABI to hold baseline data corresponding to the many fungal strains collected and studied. The database was expanded as and when new information was acquired during the project and provided a very useful common resource for all the project partners. It will be retained as it provides important information on those strains deposited for long-term storage and intended to be available for use in future studies.

WP1 Task 3 (CORI-UNIKIN CIRAD-UCL): Evaluation of the variability in isolate aggressiveness using standard inoculation tests.

To provide information on the pathogenic potential of *G. xylarioides* in support of the various laboratory-based investigations, extensive pathogenicity testing was undertaken under glasshouse and screen house conditions by project partners in Europe and Africa respectively. To facilitate this work, various host plant inoculation techniques, including treatment of roots with a spore suspension of the fungus, wounding plantlets with a spore carrying scalpel and inoculation of suspension into the stem, were evaluated and standardised methods developed. Assessment of host response to inoculation enabled representative strains of the pathogen of known aggressiveness (virulence) to be identified and included as 'markers' in subsequent trials undertaken to investigate host-pathogen interactions. The primary aims of this work were to assess host specificity and, in relative terms, aggressiveness of strains to a range of coffee species, varieties and clones and resistance within those hosts. The trials clarified the pathogenic nature of those strains assessed and confirmed the apparent specificity to a single coffee species observed under field conditions in relation to those strains associated with *C. canephora* and C. *arabica* in current outbreaks.[3] Of significance, and in contrast, historical strains exhibited a broader host range and induced CWD symptoms on coffee species other than those from which they were originally isolated. Historical strain DSMZ 62457 (=BBA 62457), for example, isolated from *C. excelsa* in CAR in 1955, exhibited pathogenicity on *C. excelsa, C. canephora* and *C. arabica* and may represent a historical link to those strains currently affecting one or other of the two coffee species. A number of the genetic approaches also clearly distinguished between all of the *Fusarium* species obtained and investigated during the project. Full details of the activities and findings of this work are reported in other sections of the Final Project Report.

WP1 Task 4: (CIRAD, CORI, UNIKIN UCL) Description of the fungal life cycle, asexual and sexual phases

The existence of defined groupings and individuals among the *G. xylarioides* strains was confirmed by biological assays and molecular studies undertaken at UCL. The teleomorph of the fungus, commonly observed on coffee trees under suitable conditions in the field, was successfully generated when two strains of opposing mating type were paired. Pairing strains from diverse origins led to the identification of three biological species (BS1-BS3), within which strains were sexually compatible, as well as a sterility group (SG4) comprising a number of reproductively sterile strains. The latter are sexually incompatible with each other and with strains in each of the BS, while the BS are sexually incompatible with each other. Correlation between the genetic and biological attributes of the pathogen was clear, in that BS1 and BS2 comprised those strains associated with *C. arabica* in Ethiopia and with *C. canephora* in DRC, Uganda and Tanzania, respectively. BS3 comprises two historical strains from Central and West Africa, including DSMZ 62457, associated with *C. excelsa*. Of importance, and given the time required to perform *in vitro* mating assays, PCR analysis (based on previously described primer pairs) and sequencing enabled the identification of mating type (*MAT*) idiomorphs (*MAT*1-1 and *MAT*1-2), confirming the hypothesis that *G. xylarioides sensu lato* is a heterothallic fungus, and will now enable mating types to be identified before confirmatory crosses are performed. Taxonomically, *MAT* sequencing and phylogenetic analysis placed the fungus within the

[3] Host specificity under natural field conditions was again apparent from the findings of surveys undertaken across eastern and central Africa in 2002-3 where, even under conditions of severe disease pressure and when the species were cultivated in close proximity, CWD was not observed on *C. arabica* in DRC, Uganda and Tanzania or on *C. canephora* in Ethiopia.

African clade of the *Gibberella fujikuroi* species complex (GFC), an important pathogenic *Fusarium* complex, and resolved four distinct phylogenetic species that correspond to the BS and SG revealed in the mating tests. These lineages could also be resolved by RAPD analysis and amplification and sequencing of a combination of non-*MAT* nuclear genes. Mating type tester strains have been identified for the four lineages and securely deposited at UCL.

The various approaches applied by the project partners proved effective in clarifying the underlying variability that exists within the CWD pathogen. Based on the molecular studies and BS/SG identified through the mating tests, and specifically in recognition of the sexually incompatible genetic lineages, four species designations have been proposed: 1. *G. abyssiniae* (anamorph, *F. abyssiniae*) sp. nov., responsible for CWD on *C. arabica* in Ethiopia; 2. *G. congoensis* (anamorph, *F. congoensis*) sp. nov., responsible for CWD on *C. canephora* in the Congo basin region (DRC, Uganda and Tanzania); 3. *F. guineensis* sp. nov., described using *C. canephora*-associated strains obtained during the earlier outbreaks in Guinea, Ivory Coast and possibly CAR; 4. *G. xylarioides* (*F. xylarioides*), neotypified using Central African *C. excelsa* strain BBA 62457 (=DSMZ 62457) and strain ATCC 15664 collected during the first CWD epidemic. The first three are previously undescribed species.

For many species of *Fusarium* it has been extremely difficult or impossible to detect and relate cultural characteristics to genetic structure or behaviour. Efforts by the various partners to discriminate strains by more conventional approaches, including morphological examination, varied in success. It was, however, possible to differentiate strains associated with *C. arabica* (i.e. BS1) from those associated with *C. canephora* and *C. excelsa* (BS2, BS3, SG4) based on production of orange pigmentation by the latter on certain agar media. Differences in teleomorphic form and in growth response to temperature were also apparent between the newly defined species. Within the *C. canephora* and *C. excelsa* strains, however, BS3 and SG4 were capable of producing colonies and pigmentation similar to those of another species, *F. udum*. At UoK, examination of cultural characteristics, including spore morphology and colony growth, enabled separation of various *Fusarium* species derived from coffee. Such investigations, while clearly providing possibilities for more rapid and in some cases straightforward identification and differentiation of fungal strains and populations, reinforce the need to exercise caution. In Africa, for example, environmental conditions (including laboratory) vary considerably and may influence the nature of the fungal response and hence any conclusions drawn. They do, however, warrant further study to confirm their suitability and, from a management perspective, to ascertain the relationship between *in vitro* response and the survival and success of the CWD pathogen in the field.

WP1 Task 5 (CIRAD-CABI-UCL): Evaluation of genetic diversity using a range of techniques including PCR and microsatellite markers

A range of fundamentally distinct molecular approaches[4] was applied principally by CIRAD, CABI, UCL and UoK to investigate underlying genetic variability within the CWD pathogen. The approaches were primarily PCR based and were intended to be complementary, enabling a study not only of the entire fungal genome but also of specific regions within the genome and, importantly, to provide an opportunity to reveal the occurrence of any artefactual results. They were selected based on their ability to reveal variability within *G. xylarioides*, to discriminate genetic groupings or populations within the fungus and to discriminate between *G. xylarioides* and other *Fusaria* from coffee.

As expected, the techniques differed in the extent to which they discriminated within and between the different *Fusarium* species. Of significance, however, several showed close correlation and were consistent in revealing the existence of a number of genetic groupings within *G. xylarioides*. The first comprised those *G. xylarioides* strains isolated from *C. arabica* affected by CWD in Ethiopia, the second *G. xylarioides* strains isolated from *C. canephora* affected in Uganda, DRC and Tanzania. The first of two major groups comprised those *G. xylarioides* strains obtained from *C. canephora* in Uganda, DRC and Tanzania after re-emergence of the disease in those countries (this group also included one strain from *C. excelsa* in Uganda). Despite the level of resolution possible with some of the molecular approaches, and while some genetic variability was observable between these two populations, no variability was detected within either of the two. As such, it was not possible to precisely define the origin of strains within each group either geographically (i.e. originating either from DRC, Uganda or Tanzania) or in terms of their coffee host. In contrast, greater variability was found

[4] Molecular approaches included analyses of microsatellite loci, presumptive mitochondrial DNA, restriction fragment length polymorphisms (RFLP), amplified fragment length polymorphisms (AFLP), inter-simple-sequence-repeat-anchored (ISSR) sequences, translation elongation factor (*tef*) and intergenic spacer (IGS) and internal transcribed spacer (ITS) regions.

between these two groups and the 'historical strains'. Indeed the latter, obtained from *C. canephora* and *C. excelsa* in the Central African Republic (CAR), Guinea and Ivory Coast in the 1950s and 1960s, could be distinguished from one another. Given the limited number of strains involved, uncertainty remains as to how and why the variability observed within the historical strains may have arisen, how it relates to field behaviour and what the relationship is between these strains and those currently affecting *C. canephoralC. excelsa* and *C. arabica*. As described below, the apparent relationship between genetic variability and coffee host is supported by pathogenicity testing to investigate the ability of individual strains to induce CWD on coffee.

WP1 Task 6 Synthesis of the results

Concluding points

- The broad ranging research completed during the course of the project provided a depth of new knowledge of diversity within the coffee wilt disease pathogen, *G. xylarioides*, in terms of recognising what appear to be four geographically defined and biologically and genetically distinct species within what was originally considered to be a single species.
- Taxonomically, these species could be placed within the *Gibberella fujikuroi* species complex (GFC) alongside other important *Fusarium* pathogens, each species representing a distinct phylogenetic (evolutionary) lineage.
- Identification of distinct mating types confirmed that *G. xylarioides* is heterothallic and enabled sexual incompatibility to be shown between the four species. It also provides an explanation for the common observance of perithecia under field conditions but its absence on coffee plants exposed to monospore cultures of the fungus.
- A clear relationship was observed between the two species associated with current CWD outbreaks on *C. canephora* (in DRC, Uganda and Tanzania) and *C. arabica* (in Ethiopia) and their ability to manifest CWD on the two coffee species. This confirmed previous suggestions of host specificity that were primarily based on observations in these countries, since re-emergence of the disease, of natural CWD development under field conditions.
- Given the apparent prevalence of the teleomorph in nature, and assuming that the perithecia so observed are fertile, the limited genetic variability detected within the four species, particularly those responsible for current outbreaks in eastern and central Africa, is somewhat surprising. This may be due to selection pressure imposed by widespread planting of coffee of limited genetic diversity and/or the relative fitness and rapid spread of the few genetic variants identified.
- It is apparent from the research that the historical strains are not directly responsible for the current epidemics in eastern and central Africa, although their historical role, if any, remains unclear. Furthermore, it is unclear what role they had in the earlier widespread and very destructive outbreaks in western and central where other, as yet unidentified, variants may have been involved.

Implications for CWD management

The project findings are crucial to future management of CWD. In the first instance, the observation that only two genetically homogeneous and sexually incompatible variants of restricted host range are responsible for current outbreaks is theoretically beneficial. By various means, *in vitro* and *in vivo,* these may now be accurately identified and monitored, while prospects for developing and utilising new and more effective and reliable 'tools' for diagnosis and identification are improved. This is critical from a quarantine perspective and in terms of our ability to monitor disease spread, avoid inadvertent introduction of the pathogen and detect emergence of new variants. Given the losses already incurred by coffee producers in eastern and central Africa, measures must be implemented to contain the two species currently present in these areas and thereby protect cultivation of *C. canephora* and *C. arabica* in other areas. The associated possibility of new variants that are more aggressive and capable of attacking a broader range of coffee, arising through possible sexual recombination should these two forms come into contact, must also be avoided. Where agronomic conditions are favourable, the knowledge and tools generated by the project will also inform on the deployment of coffee of known resistance or tolerance to tackle variants already present in, or presenting a threat to, specific areas of production. Such a strategy may be effective over large areas and remain relatively stable and sustainable, given the widespread occurrence and uniformity of the species currently associated with *C. canephora* and *C. arabica*. Project outputs may also be adopted to improve and accelerate ongoing breeding programmes, and ensure that they adequately address local variability, and to facilitate in-depth studies of CWD epidemiology and host-pathogen interactions.

WP2 - Host/Pathogen interaction

WP2 Task 1 (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.

Different standardized artificial has been developed by each partner, adapted to local plant material procurement and manpower constraints.

At CORI, different methods of inoculation have been evaluated. The root dipping method is currently the method in use for screening CWD resistance. Young plants aged 9 to 12 months grown from seeds or rooted cuttings, were artificially inoculated by root dipping using a mono-conidial local inoculum of *F. xylarioides* isolated from infected stem parts of *C. canephora* clone 257s/53. The plants were stripped off all planting medium (soil) and their roots were washed with tap water before dipping the entire root section for 30 minutes into a water suspension inoculum at $1.0x10^6$ conidia ml^{-1}. After inoculation, the plants were replanted in polythene pots filled with sterilized soil enriched with organic nutrients. Re-planted plants were kept in the screen house for incubation at room temperature. The plants were regularly watered with tap water as they were being monitored for CWD symptoms.

At CIRAD, seedlings aged 10 to 12 weeks were inoculated with a water suspension inoculum at $1.0x10^6$ conidia ml^{-1}, derived from a single conidium of *F. xylarioides* isolate CAB003 collected on C. canephora in Uganda. Young plants were inoculated by wounding under the cotyledonary leaves with a scalpel and applying 1-2 drops of the inoculum into the wound using the scalpel. Inoculated plants were incubated in a room with the temperature, relative humidity and light regulated at 25°C, 80% and 12h/12h lighting regime, respectively. The plants were watered twice a week with tap water using a hosepipe as they were being monitored regularly for wilt symptoms.

At UNIKIN, plants aged 9 to 12 months grown from seeds cultivated in a screen house (25°-28°C) were inoculated with a syringe, by a drop of a spore suspension adjusted to 1.10^6 conidia/ml injected into the stem.

Young plants were inoculated above the cotyledonary leaves (first internode). After inoculation they were kept in the screen house under shaded conditions.

Every partner checked the validity and repeatability of the results and a reference isolate was identified for the artificial inoculations.

WP2 Task 2 (CORI-UNIKIN) Conduct similar field inoculations in Africa to validate the inoculation method

In Uganda, *C. canephora* clones were assessed for resistance in the field and the results were compared with results of artificial inoculation obtained by different inoculation techniques on clones and open pollinated seedlings in a screen house at Kituza and a climatic chamber at CIRAD.

Field resistance was confirmed by artificial inoculation performed on rooted cuttings in the screen house at CORI, although disease levels on the clones were not exactly the same. Moreover, the correlation between mortality among clones in the field and in the screen house (rooted cuttings) was significant ($p=0.006$). This indicates that field and screen house assessments give comparable resistance results and both protocols can reliably be used for assessing resistance to CWD in *C. canephora*.

It was observed that traits/indicators of resistance to coffee wilt disease (% of dead plants, % of defoliation, AUDPC) considered in this study were explicitly expressed in the field, green house and under controlled climatic conditions. This shows that field, screen house and climatic chamber conditions allow appropriate expression of this disease therefore making it possible to distinguish the resistance of different genotypes.

It was also observed that these traits were explicitly expressed irrespective of the infection technique. These results illustrate that infections through root dipping, scalpel wounds and stem wounding by drilling are effective and give results that are comparable to field infection. Inoculation by root dipping is adopted for assessing resistance to coffee wilt disease among germplasms of different coffee species (*C. canephora*; *C. arabica*, Arabusta) and plant material types (rooted cuttings or seedlings) at CORI (Musoli et al., 2001). Wounding by stem drilling is an inoculation technique used at CIRAD and scalpel stem wounding is a technique adapted for studies on arabica (Girma and Hindorf, 2001). For all types of infections, results were obtained quickly and at far less cost compared to field assessment. This implies artificial inoculations can be used to reduce the time and cost of initial assessment of resistance and are convenient for preselection tests.

WP2 Task 3. Analysis of host specificity interaction

To develop a breeding programme it is necessary to define host/pathogen interactions. These studies are possible outside a coffee producing country.

All the isolates collected from the different species of *Coffea* were assessed in crossed inoculations.

Canephora strain CAB003 was specific to the species *C. canephora*.

Arabica strain CAB007 seemed specific to the species *C. arabica,* despite the 5% mortality obtained with a progeny on *C. dewevrei* and *C. canephora.* The specificity of isolate CAB007 to the species *C. arabica* therefore seems to have been confirmed in the growing zones where both species exist side by side. In Ethiopia in the Jima zone, at the EARO research centre in a severely contaminated area, only *C. arabica* plants were affected, with *C. canephora* plants remaining unaffected.

Isolates CBS74979 and CBS25852 from Guinea confirmed their pathogenicity on the species *C. canephora*. They also proved to be pathogenic on the species *C. dewevrei,* which is not the species from which those strains were collected.

Although isolates ATCC15664 and DSMZ62457 were isolated from *C. dewevrei,* they were pathogenic on the two species *C. canephora* and *C. liberica*.

Those historical isolates tested displayed a broad host spectrum, that could induce young plant mortality in both species, *C. canephora* and *C. dewevrei,* and isolate DSMZ62457 was also pathogenic on the 3 species.

Although these results are only partial. It was quite complicated to proceed with a complete crossed inoculation due to the lack of *C. liberica* seeds in sufficient quantities. They nonetheless show that the historical isolates had a broader host spectrum than the contemporary population represented by isolate CAB003.

The interactions are summarized in table 1.

	CAB003	CAB007	DSMZ62457	CBS25852	CBS74979	ATCC15664	ATCC36325
C. canephora	+++	-	+	+	+	+	-
C. liberica	+		+++	+	+	+	-
C. arabica	-	++	++				-

These results highlight the evolutionary potential of *F. xylarioides*. While the contemporary isolates are specific to a species, the probability of the contemporary population evolving towards the acquisition of a broader host spectrum cannot be ruled out. This means that the selections currently in progress should be assessed in relation to their specific isolate but also in relation to isolate DSMZ62457.

WP2 Task 4 Combine all these observations to propose a hypothesis on the nature of the resistance

At CORI, resistance of *C. canephora* genotypes to CWD in the field showed a percentage mortality range of 0 to 96%. Disease period (duration from appearance of first symptoms to death of the tree) was also variable between clones, ranging from one to 16 months. Generally resistant genotypes had long disease periods, however certain susceptible genotypes (Q/1/1) had exceptionally long disease periods and also certain resistant genotypes (R/1/4) had very short disease periods. These observations suggest there is variation in resistance mechanisms among the genotypes, which implies that different genes condition the resistance and in different ways and thus further supporting the hypothesis of resistance to CWD in *C. canephora* being polygenic. But with clones J/1/1 and Q/3/4 being completely resistant to this disease in the field, the nature of resistance can be confirmed after an analysis of progenies derived from specific crosses of resistant/susceptible genotypes.

A comparison of CWD resistance among *C. canephora* genotypes in the field and under artificial inoculations in the screen house and climatic chambers revealed that field, screen house and climatic chamber results are comparable and convenient for pre-selection tests. However, a differential reaction was observed with clone B2/1 between aerial inoculation by stem wounding and inoculation by root wounding (root dipping). This suggests there are different mechanisms of resistance expressed by the ability of the plant to resist penetration of the fungus either through roots or aerial parts. Bugbee and Sappenfiled (1968) reported a similar resistance mechanism for *Fusarium oxysporum* f. sp. *vasinfectum* in Cotton (*Gossypium hirsutum*).

The findings from artificial inoculations show that some accessions are completely resistant to *F. xylarioides*. The resistant plant material is not specific to one genetic group.

The inheritance of resistance factors will be pursued in further experiments as part of a new artificial pollination plan.

WP3 - Breeding for resistance

WP3 Task 1: Identify sources of resistance through field assessment

Analysis of disease data on *C. canephora* half sib progenies from different populations (wild plants from the Itwara and Kibale forests, feral trees from the Kalangala islands isolated on Lake Victoria and cultivated trees of the Erecta and Nganda sub-groups) revealed that Kalangala and Itwara populations were highly resistant to CWD, highlighting the importance of these populations as sources of resistance genes for inclusion in the commercial varieties. The Nganda and Erecta populations were very susceptible, while Kibale progenies were moderately susceptible. Significant variability was also observed within each of these populations.

C. canephora genotypes in the germplasm collections at CORI and trees surviving among farms devastated by CWD were artificially tested in the screen house for resistance. Over 1500 resistant individual trees were identified and they have been planted in mother gardens for multiplication and field evaluations (for resistance to diseases, quality and yield) as clones.

Artificial tests performed on seedling progenies of various *C. arabica* varieties found this species to be resistant to CWD in Uganda. Similar tests performed on F1 Arabusta (*C. arabica* x *C. canephora*) clones found the Arabusta genotypes to be resistant to CWD in Uganda. This confirmed that CWD is not a problem for *C. arabica* and this species together with the F1 Arabusta can be used as sources of resistance genes to control CWD of *C. canephora* in Uganda.

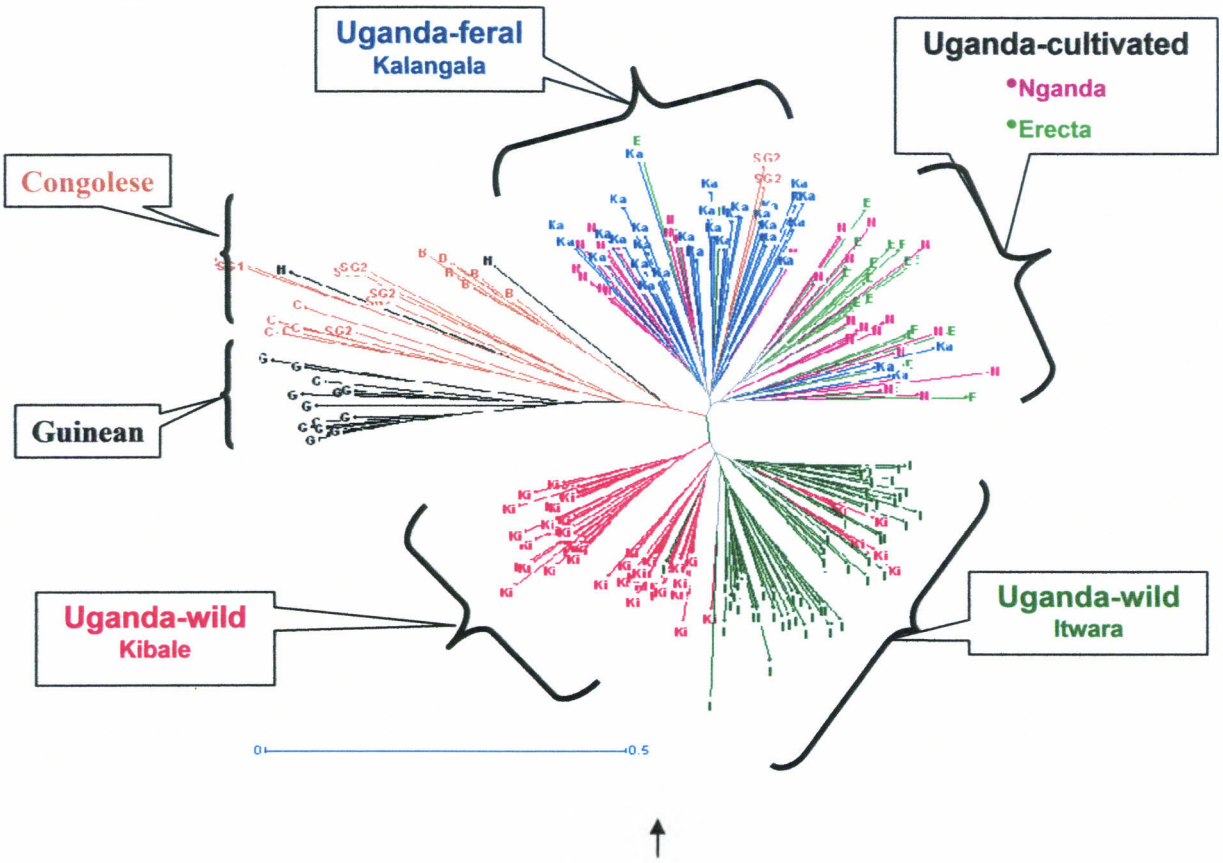
WP3 Task 2: Collect seeds from genotypes representative of the available germplasm, dispatch them for screening.

In order to carry out studies on the genetic diversity of *Coffea canephora* and also assess resistance, it was essential to have individual plants representing the maximum genetic diversity known in this species. To achieve that, different contributors took part in these exchanges and different sources were asked for. First of all, the existing genetic resources were called upon:
- the collections of the two partners, CORI and UNIKIN,
- the NARS in Ivory Coast, which accepted and exchange of planting material and its evaluation
- CIRAD, which used some of the genetic resources preserved in French Guiana
- CATIE (Costa Rica), which supplied seeds of different *Coffea* species for the analysis of interactions.

Then, in order to maximize the genetic diversity studied, surveys were made of unaffected coffee trees in Uganda and DRC in high-pressure zones.
Lastly, surveys were made of coffee trees in the primary forests of Uganda, to enhance our knowledge of the genetic structure of this species.

Berthaud (1986) first identified two diversity groups, *a* Congolese group, which comprised genotypes from CAR, Congo basin and Cameroon and a Guinean group, which consisted of genotypes of wild origin from Ivory Coast. Montagnon et al. (1992), proposed a substructure in the Congolese group with two sub-divisions SG1 & SG2. Dussert et al. (2003), placed *C. canephora* genotypes of cultivated and wild origins into five diversity groups, adding two groups B and C to the Congolese group. Recent studies using SSR markers on *C. canephora* among other coffee species (Poncet *et al.* 2004) and on *C. canephora* alone (Cubry et al. 2005) confirmed the structure described by Dussert et al. (2003).

Representative genotypes of wild coffee from primary forests were analysed using 24 microsatellite markers. The accessions were collected from 5 populations in the Kibale forest and 6 populations in the Itwara forest and from 4 populations in the Kalangala islands forest (feral population). Representative genotypes of the known Guinean and Congolese *canephora* groups were included as controls. Eighteen of these markers were used to compare Ugandan populations with previously known genetic diversity groups of the species. Ugandan *C. canephora* was found to be highly diverse with a genetic sub-structure consisting of wild, feral and cultivated populations. Ugandan *C. canephora* was found to be different from previously known diversity groups, implying that it forms another diversity group within the species.



WP3 Task 3: Conduct screening tests in both Africa and Europe using isolates with a wide range of aggressiveness.

There are significant genetic differences for resistance between different Ugandan *C. canephora* populations (Kalangala islands, Itwara and Kibale primary forests, Nganda and Erect phenotypes, plant material from hot spots). This suggests that genes for CWD resistance are differently distributed among *C. canephora* populations in Uganda. The Kalangala (Island) and Itwara (wild forest) populations which were found to be most resistant are anticipated to have the highest number of resistant genotypes and or resistance genes. High levels of resistance observed among Kalangala (Island) and Itwara (wild forest) populations highlights the need for exploring different *C. canephora* germplasm sources for resistance to develop commercial CWD resistant varieties. It also highlights the likelihood that these populations are valuable sources of genes for improving other agronomic traits of *C. canephora* varieties.

Nevertheless, high within-population variability was observed. Kalangala and Itwara, which were overall the most resistant populations, had the highest within-population variations. This highlights the need for conducting CWD tests and targeting resistant genotypes in populations for resistance to develop CWD resistant varieties. This further confirms variable distribution of resistance genes among *C. canephora genotypes*.

At CIRAD, *coffea canephora* accessions from the different genetic groups were evaluated. Coffee trees displaying infected plant rates below 10% were considered in the first analysis to be tolerant and possibly a source of genes of resistance for a breeding programme. Those results needed to be confirmed by a new series of artificial inoculations and verification in the field in an infested zone.

The resistance of the coffee trees belonging to the Guinean group was confirmed. Tested coffee trees from that group displayed infected plant percentages ranging from 0 to 5%. However, a few Guinean populations exceeded that percentage.

It was not possible from these exploratory results to identify a genetic group that was more likely to bear resistance factors. A few resistant progenies were found in all the groups.

Moreover, progenies found to have a mortality rate of 0% or under 5% will have to be confirmed and will be selected as a source of resistance to Coffee Wilt.

In DRC, a different level of resistance was observed in plant material from Luki, Kiyaka.

These results show that there exist sources of resistance that can be detected by early screening. Resistance needs to be confirmed in a natural infection zone before using these genetic resources in a breeding programme.

WP3 Task 4: Establish multi-site field trials of wilt resistant/tolerant varieties.

At CORI, among the genotypes studied, clone J/1/1 was completely resistant to CWD in the field and clone Q/3/4 was highly resistant. The two clones are known to yield on average 3.3 and 2.7 metric tons (five year average) of clean coffee per hectare per annum, respectively. Since they were initially selected for having good quality and being resistant to leaf rust and red blister disease, it implies they almost have all traits required of a commercial variety and they should be readily available for mass propagation. But as their initial assessments were carried out at only one site (Kituza), it is apparent that the quality, yield and other important traits need to be validated in multi-site trials in different agroecological *C. canephora* growing areas within the country.
It is also apparent that two clones are not enough for massive replanting in all *C. canephora* growing areas within Ugandan for two reasons: first, host resistance depends on host, pathogen and environmental interactions. Therefore it is not obvious that the two clones will be resistant to the same levels if they are cultivated in agro-ecological conditions that are different from those at Kituza, especially if the resistance is partial/horizontal as observed on clone Q/3/4 in the field and on both clones in the screen house. Thus, these clones will have to be re-evaluated for CWD resistance in on-farm multi-site trials under different agro-ecological conditions before they are released to the farming community. Secondly, large-scale coffee growing based on only two clones will be vulnerable in the event of another disease or pest outbreak. Also, due to the out-breeding nature of *C. canephora,* it is recommendable to plant at least five different clones for successful pollination and fruiting, hence good yields. To avoid such risks, in the short run, the best of the moderately susceptible clones such as R/1/4 and commercial clones 258s/24 and 1s/3 should be included to broaden the genetic base, as more resistant clones are awaited from the germplasm screening programme.
Therefore, the first activity will be to multiply CWD-resistant clones identified in these studies for validating their performance in different *C. canephora* agro-ecological areas within Uganda. The clones will be multiplied either as rooted nodal cuttings or tissue culture plantlets generated through somatic embryogenesis or by both methods. The facilities and expertise for both multiplication methods are available in Uganda.

WP3 Task 5: Study inheritance of CWD resistance

Analysis of disease data on *C. canephora* half diallel progenies found significant general combining ability for CWD susceptibility/resistance, implying there is an additive genetic relationship between parents and offspring for CWD resistance. Broad sense heritability estimated from the same progenies at a disease level of 55-65% plant mortality was moderate (0.27-0.33) and corresponding narrow sense heritability was low (less than 0.15). Broad sense heritability estimated from an array of clones, within the same disease range, was medium (31-35). This shows that CWD resistance is heritable and therefore progenies of crosses between susceptible and resistant parents are expected to have better resistance than their susceptible parents. Thus resistance of current Ugandan commercial clones, which are susceptible to CWD, can be improved through hybridisation with resistant genotypes. Bearing in mind that *C. canephora* is predominantly out-breeding and these clones are heterozygous, progenies of theses crosses are expected to be heterogeneous. Therefore it is not likely that all individuals in a progeny of a cross between resistant and susceptible parents will have CWD resistance to equal and acceptable levels and at the same time retain all qualities of the commercial parent. The progenies can therefore be evaluated as individual trees for all traits equally and resistant individuals, which have the other traits within acceptable limits, will be selected. These individuals will be multiplied as clones for assessment in multi-site field trials before they are released to farmers.

WP3 Task 6 Propose a breeding strategy for durable resistance

Hybridization programmes

Although CWD has taken precedent, yield and quality are very important aspects of the coffee variety improvement programme in Uganda. Hence specific crosses of CWD resistant genotypes with the current commercial clones will be carried out to combine CWD resistance with the high yields (2.5 tons of clean coffee per hectare per annum) (Kibirige et al, 1993), good bean qualities (18-22 g hundred-bean weight, over 90% retained by screen 18/64) and good cup qualities of the commercial clones. *C. canephora* genotypes are predominantly out-breeders and highly heterozygous, progenies of these crosses are therefore expected to be very heterogeneous. Therefore it is not likely that the entire progeny of a cross will have improved CWD resistance to acceptable levels and at the same time retain all qualities of the commercial parent. Progenies will therefore be planted and evaluated in on-station field trials as individual trees for all traits equally and genotypes within acceptable limits will be selected. Selected individuals will be multiplied as clones and planted in multi-site trials for re-assessment for adaptation and adoption. Clones that perform well will be selected for release to farmers as clonal varieties.

Hybridization will also be conducted involving CWD resistant parents with complementary traits. Such hybrid progenies will be evaluated as individual trees for resistance to CWD and their field performance for other traits. Progenies that perform well will be selected, multiplied vegetatively and planted in multi-site trials for adaptation and adoption tests. Clones that perform well will be selected and released to farmers as clonal varieties.

Where entire progenies of resistant/resistant parents perform well, parents of such progenies will be planted in polycross seed gardens for production of seeds to be given out to farmers. It is anticipated that progenies involving parents from different populations will benefit from hybrid vigour derived from double heterozygosity of its parents.

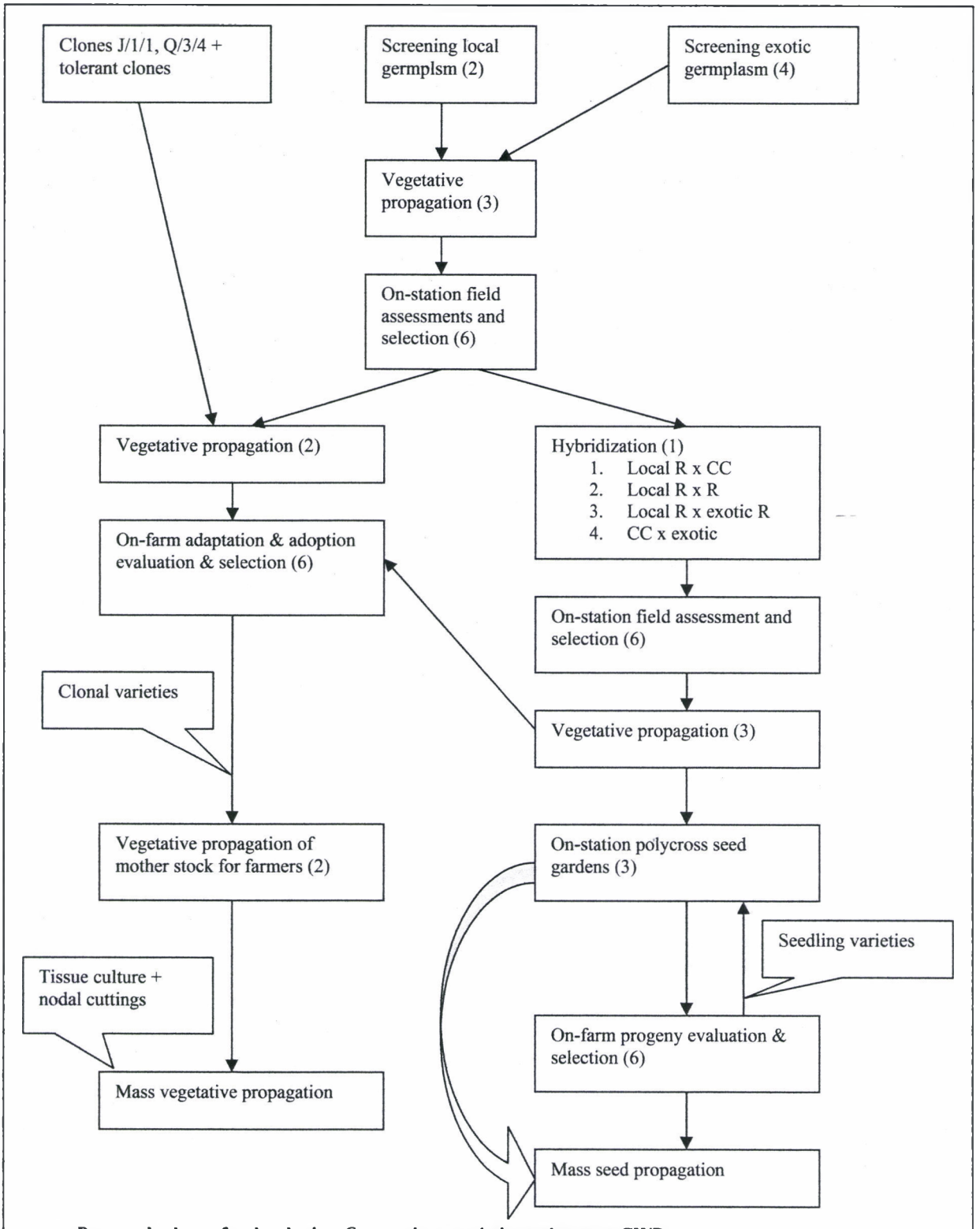
Based on field reports from Uganda and results of previous artificial inoculations (Musoli et al., 2001; Girma and Hindorf, 2001,) *C. arabica* can also be a source of resistance for controlling coffee wilt disease in *C. canephora*. Although *C. canephora* and *C. arabica* interspecific hybrids (Arabusta) have been reported, we should anticipate some difficulties in hybridization because of the difference in ploidy levels of the two species. Crosses will be carried out between *C. arabica* and *C. canephora* artificial tetraploids. Cytological analysis will be performed on root cells of the F1 seedlings to verify true tetraploid individuals before they are planted out in the field. The true tetraploid F1 progenies will then be planted and evaluated for resistance to CWD and other agronomic traits in on-station field trials. Individual genotypes with CWD resistance and *C. canephora* cup qualities will be selected and vegetatively multiplied for evaluation in on-farm trials. Clones that perform well at this level will be selected and released to farmers as clonal varieties.

Integrated coffee wilt management

As the activities proposed above continue, and bearing in mind that none of them will give a new variety in less than five years, a short-term option will be to continue providing farmers with planting materials of the less susceptible commercial clones. From this study it was established that commercial clones 257/53, 1s/6 and 1s/2 are very susceptible. These clones should be withdrawn from the commercial list. Only clones 223/32, 258s/24 and 1s/3 should be given out. These clones should be planted in newly cleared fields expected to be free of CWD infection. Wilting plants in these new plantings should be rouged out as according to observations in this study.

Conservation of C. canephora genetic resources

Bearing in mind the ongoing degradation of natural biodiversity reserves, it is important that the *C. canephora* diversity observed in this study be protected. Since genetic differentiation among *C. canephora* populations is high, samples of a few genotypes will be obtained from many populations to capture enough natural genetic variability for conservation and utilization in future breeding programmes. Ugandan *C. canephora* samples will be collected from Itwara, Kalangala and sites in Kibale in addition to other relict forests and isolated cultivated regions not included in this study. Because of threats from coffee wilt disease and other unforeseen natural disasters on *C. canephora* genetic resources, efforts will be made to find a collaborator or an international germplasm collection for conservation in multiple conservation centres in different countries to preserve them.



Proposed scheme for developing *C. canephora* varieties resistant to CWD
Figures in brackets indicate the time in years; R = resistant; CC = current commercial varieties

WP4 - The disease: epidemiology

WP4 Task 1: Identify sites to characterise climate soil, variety, where the epidemic can be surveyed during the project.

In Uganda, on-farm observations started in 2002 on the development and spread of CWD on selected smallholder coffee farms in Mayuge and Iganga in Eastern Uganda and Masaka and Rakai in Central Uganda 2 farms per district. Visits to sites for data collection were made once at 4 or 8 weekly intervals. Wilt incidence and severity were recorded in marked areas (8 rows x 16 rows = 128 trees) for observations. The spatio-temporal spread to field scale on the coffee research station started in April 2001.

In DRC the survey started at the beginning of the project in the different province affected by the wilt.

WP4 Task 2 (CORI, UNIKIN): Description of the spatio-temporal spread of the disease.

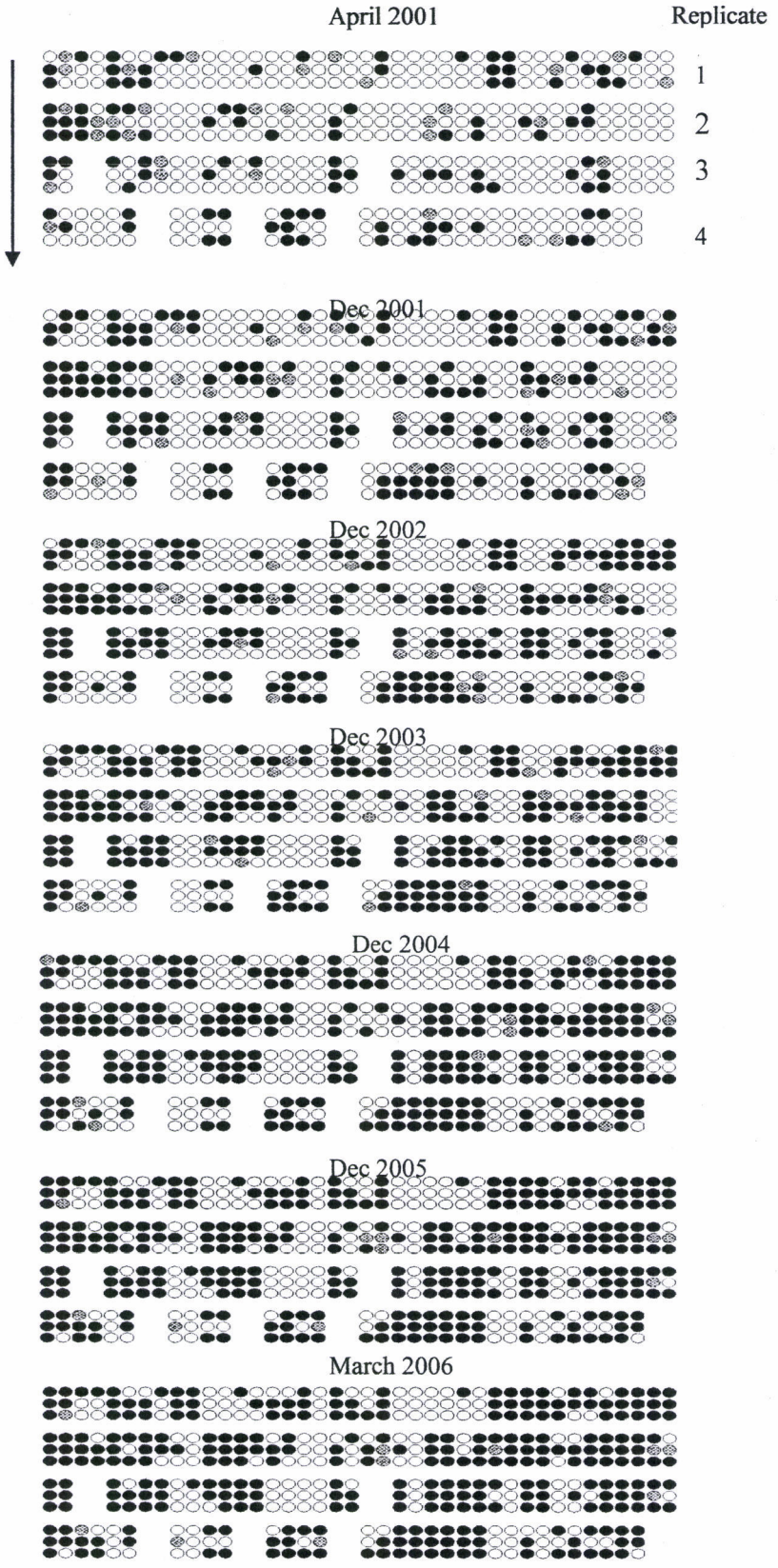
Analysis of disease data collected from *C. canephora* trees in fields at the Coffee Research Institute-Kituza in Uganda revealed that coffee wilt disease spreads gradually and continuously among *C. canephora* gardens as it kills all susceptible trees. Since CWD emerged in experimental fields where these studies were conducted in 1999, overall tree mortality progressed from 0 to 65%. However, on some varieties the disease progressed up to 96% mortality, while on others mortality remained at 0%.

Disease progress curves showing temporal disease development and maps of diseased trees were generated from the disease data. Semi-variance analysis and kriging were performed on the data to show the spatial-temporal structure of disease. Host influence on the spatial-temporal structure was deduced through distribution pattern of diseased and healthy trees and analysis of variance. Results show that the disease epidemic progresses gradually overtime. The disease was found to spread irregularly from initial infections to healthy neighbour trees, leading to aggregated patterns. An infected tree can infect up to three healthy trees away, in any direction. Disease foci form and grow with time, coalescing to one continuous stretch, only punctuated in spots planted with resistant hosts. There were varying levels of susceptibility among host genotypes, exhibiting varying rates and levels of disease development.

The study shows that at early stages of CWD epidemic, a more or less random distribution pattern was observed. In time, clusters of diseased and dead plants were formed, expanding in all directions. The high structural or sample variance derived from semi-variance analysis revealed that the spatial dependence for the disease spread is high, which implies that plant to plant infection plays a major role in CWD spread. However, the actual method(s) by which CWD spreads e.g. through root contact between diseased and healthy trees, by ascospores or conidia; is still unclear.

The disease progress curve illustrated that once CWD has invaded a *C. canephora* field, it will continue devastating the coffee trees until all susceptible trees are affected and will thus eventually die. The epidemic rate was reduced drastically as tree mortality exceeded 50%, since the number of susceptible trees had greatly reduced.

The variability observed in disease progression and final disease levels on the *C. canephora* clones in this study illustrate that host genotypes influence spatial and temporal CWD development.



Pattern of diseased trees

Arrow is pointing down slope; Each tree is represented with a circle; Black circles are trees killed by CWD, Circle with cross squares show sick tree and white circles show uninfected trees; 4 replicates in the field separated by larger clear lines; 2 rowsx3 columns of circles in each replicate represent a clone; White gaps within replicates represent missing data; Trees uprooted prior to April 2001 are included among the dead

WP4 Task 3& 4 (CORI, UNIKIN) Evaluation of the duration of the survival form of the pathogen and the ways and duration of survival of the pathogen in the field.

It is apparent from this study that disease spread within the field can occur from one infected plant to the next probably through root contact. Dry wood in the field is the major source of inoculum, which forms abundantly in the form of perithecia. Human activities can accelerate disease spread within the field and beyond. This information can be used to restrict people from moving plant parts.

Many times farmers have asked how long the pathogen persists in the soil, in infected wood pieces or if it can be transmitted through the use of common tools on the farm. This task has provided the information. The pathogen can persist in soil and wood for up to 6 months. Farmers are advised to uproot and burn infected plants. Soil for nursery use should be well sterilized and tools commonly used in the field should be sterilized by flaming or other means.

Information collected on the life cycle of the pathogen is useful for formulating control measures. We are aware that perithecia are a major source of inoculum, which forms on drying wood. This further strengthens the recommendation to burn all infected plants.

WP4 Task 5: Elaboration of a simplified model of the epidemic and proposal of adapted recommendations.

These observations imply that, for effective control of coffee wilt disease by uprooting infected trees, up to 3 healthy looking trees neighbouring a diseased tree should also be uprooted on first observation of CWD symptoms. This can also be considered precautionary given that the disease incubation period is normally long (Saccas, 1951, 1956) and therefore the healthy looking trees could be infected. Secondly, the actual mechanism of tree to tree infection is not known (either by water run off, splashing, canopy contact or root contact) and therefore a three coffee tree distance ensures that uninfected trees are out of range from this obvious inoculum source.

The practical implication of these results is that this intervention is only limited to low disease levels, at probably 5% incidence. At higher disease incidence, there will be too many diseased trees and uprooting becomes too costly (In Uganda it costs about US $0.5 to uproot a diseased coffee tree). Secondly, at a disease incidence of more than 5% there are many infection points and uprooting 3 trees in all directions from each of those initial points will result in uprooting a large proportion of the crop, leaving the field virtually devoid of coffee trees. Lack of such information may have contributed to the failure of uprooting intervention in Uganda (Wetala et al., 2000), where uprooting trees with disease symptoms was emphasized irrespective of disease incidence in the field.

PROBLEMS ENCOUNTERED

Technical problems encountered

Transfers of *C. canephora* plants between Uganda and CIRAD required a material transfer agreement between CIRAD and NARO, for which the legal process was lengthy and caused a delay in this activity for more than one year.

Secondly, some activities, particularly artificial pollinations (hybridization) are dependent on prevailing weather conditions. The extreme and prolonged dry season experienced between March and September 2004 prevented flowering and consequently there were very few artificial pollinations during that period. This caused a shortfall in the number of hybrid seedlings available for screen house studies on the inheritance of CWD resistance. Therefore there was a delay in this activity and consequently we requested a one-year extension to the project, to allow for this activity.

Administrative problems encountered

The major problem encountered concerned the constraints experienced by several partners in carrying out the planned research, mainly due to financial problems. In a lot of cases CIRAD funded part of the annual budget in advance.

Excessively long delays in the submission of financial reports or/and scientific reports which consequently prevented the release of funding.

During the project, the partners experienced some difficulties in interacting with partner P3 to establish scientific activities on plant pathology aspects concerning the sexual cycle, survival of the pathogen and spread of the disease in on-farm experiments.

Technology implementation plan

The fundamental result of the COWIDI project is a structural analysis of the *Fusarium xylarioides* population responsible for Coffee Wilt Disease and a clearer understanding of *Coffea canephora* resistance, with an enrichment of *C. canephora* germplasm, the identification of resistance sources in existing genetic resources, or collected over the project period.

No "product" suitable for direct commercial development was produced during the project.

The results obtained have been or will be published and will be accessible to the public.

PUBLICATIONS - CONFERENCE

Thesis

Lepoint, P.C.E. December 2006. Speciation within the African coffee wilt pathogen. Thesis. Université catholique de Louvain. 208pp.

MUSOLI Chungason Pascal, Mars 2007. Recherche de sources de résistance à la trachéomycose du caféier *Coffea canephora* Pierre, due à *Fusarium xylarioides* Steyaert en Ouganda. Docteur en Sciences, de l'Ecole Nationale Superieure Agronomique de Montpellier

Publications accepted

Tshilenge-Djim, P., F.T.J. Munaut, A. Kalonji, and H.M.M. Maraite. 2004. Caractérisation des *Fusariums* spp. associées au dépérissement du caféier Robusta en République Démocratique du Congo. Parasitica 60: 67-82.

Lepoint, P.C.E., F.T.J. Munaut, and H.M.M. Maraite. 2005. *Gibberella xylarioides* from *Coffea canephora*: a new mating population in the *Gibberella fujikuroi* species complex. Appl. Environ. Microbiol. 71 (12): 8466-8471.

RUTHERFORD, M. (2006). Current knowledge of coffee wilt disease, a major constraint to coffee production in Africa. *Phytopathology* 96, 663-666.

Papers submitted

Pascal Musoli, Philippe Cubry, Pauline Aluka, Claire Billot, Magali Dufour, Fabien De Bellis, Denis Kyetere, James Ochugo, Daniel Bieysse, André Charrier and Thierry Leroy. A new genetic diversity group from Uganda within *Coffea canephora* Pierre. Submitted to the Genetics Journal

Pascal C. Musoli, Christian Cilas, David Pot, Agnes Nabaggala, Saleh Nakendo, James Pande, André Charrier, Daniel Bieysse, Thierry Leroy. Inheritance of resistance to coffee wilt disease *(Fusarium xylarioides* Steyaert) in *Coffea canephora* Pierre. Submitted to New phytologist Journal

Pascal C. Musoli, C. Cilas, A. Charrier, A. Kangire, G.M. ten Hoopen, C. Kabole, J. Ogwang[1] and D. Bieysse. Spatial and temporal analysis of Coffee Wilt Disease caused by *Fusarium xylarioides* Steyaert in *Coffea canephora*. Submitted to European Journal of Phytopathology

Lepoint, P.C.E. Characterization of the *MAT1-1* and *MAT1-2* idiomorphs in the *Gibberella xylarioides* and *Gibberella indica* Species Complexes (*In preparation for Fungal Genetics and Biology*).

Lepoint, P.C.E., C.A. Decock, F.T.J. Munaut, and H.M.M. Maraite. Speciation within the *Gibberella xylarioides* and *Gibberella indica* Species Complexes (*In preparation for Fungal Genetics and Biology*).

Lepoint, P.C.E., C.A. Decock, F.T.J. Munaut, and H.M.M. Maraite. Neotypification of *Gibberella xylarioides* (*Fusarium xylarioides*) (*In preparation for Taxon*).

Conference papers or/and oral presentation

RUTHERFORD, M.A., INESON, J., BUDDIE, A. and FLOOD, J. (2002). Regional Coffee Wilt Programme: CABI UK Centre research activities. Regional Coffee Wilt Programme Stakeholders' and Planning Workshop, Nairobi, Kenya, 13-14 November 2002 (presented by M. A. Rutherford, CABI E-UK).

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

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

FLOOD, J. (2003) Fusarium wilts of tropical perennial crops- an overview of management strategies: Challenges to the management of *Fusarium* wilts of tropical crops. International Fusarium Wilt Symposium, Salvador, Brazil Sept. 2003 (presented by J. Flood, CABI E-UK).

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

Pinard Fabrice, Bieysse Daniel, Leroy Thierry, Dufour Magali. Regional Coffee wilt Programme stakeholders and planning Workshop. "Breeding strategy for CWD resistance based upon CWD diversity". Nairobi, Kenya. 9-13 November 2004

Janzac Bérenger, Roussel Véronique, Bonnemaire Katia, Hakiza Georgina, Kalonji Adrien, Pinard Fabrice, Bieysse Daniel. Etude de la biodiversité de *Fusarium xylarioides* agent de la trachéomycose des caféiers. Implications dans le schéma de sélection de *Coffea canephora*. 5eme rencontres de Phytopathologie. 18-22 janvier 2004 Aussois France.

JANZAC Bérenger, MUSOLI Pascal, ROUSSEL Véronique, BONNEMAYRE Katia, PINARD Fabrice, LEROY Thierry, DUFOUR Magali, KYETERE Denis, HAKIZA Georgina, TSHILENGE Patrick, KALONJI Adrien, GIRMA Adugna, BIEYSSE Daniel. Control of Coffee Wilt: study of genetic diversity *Fusarium xylarioides* and *Coffea canephora* in Uganda. 20th International conference on Coffee Science. 11-15 Octobre 2004 Bangalore India.

Hakiza, G.J, Birikunzira, J.B., Olal, S. and Kabole C. "Preliminary studies on the epidemiology of Coffee Wilt Disease (Gibberella xylarioides) in Uganda. Poster presentation. Proceedings of the 20th International Conference on Coffee Science, ASIC, Bangalore, India, October 11 – 15, 2004

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, CABI E-UK).

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

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

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

RUTHERFORD, M.A., HAKIZA G ' ABEBE M, GIRMA A., KILAMBO D., BIEYSSE D.(2006) Epidemiology and variability of the coffee wilt pathogen. pp 42-44 in: Sweetmore,A., Kimmins, F. and Silverside, P. (eds). *Perspectives on Pests II: Achievements of research under UK Department for International Development Crop Protection Programme 2000–05.* Natural Resources International Limited, Aylesford, UK. 260+xvi pages. ISBN: 0-9546452-7-8

KILAMBO, D. L., NG'HOMA, N. M., MTENGA, D. J., TERI, J. M., NZALLAWAHE, T., RUTHERFORD, M. and MASUMBUKO, L. (2006). Progress towards searching for durable resistance to Fusarium Wilt (*Fusarium xylarioides*) in *Coffea canephora* germplasm in Tanzania. *Proceedings of the 21st International Scientific Conference on Coffee Science* (ASIC), 11–15 September 2006, Montpellier, France. pp 1386-1389

GIRMA, A.S., FLOOD, J., HINDORF, H., BIEYSSE, D., SIMONS, S. and RUTHERFORD, M. (2006). Tracheomycosis (*Gibberella xylarioides*) - a menace to world coffee production: evidenced by cross inoculation of historic and current strains of the pathogen. *Proceedings of the 21st International Scientific Conference on Coffee Science* (ASIC), 11–15 September 2006, Montpellier, France. pp 1268-1276

Pascal Musoli, Pauline Aluka, Philippe Cubry, Magali Dufour, Fabien de Bellis, James Ogwang, Denis Kyetere, Thierry Leroy, Daniel Bieysse, André Charrier. Fighting coffee wilt disease: Uganda wild *C. canephora* genetic diversity and its usefulness. ASIC 11-16/09/2006. 21st International Conference on Coffee Science, Montpellier, France

Bieysse, D., Lepoint, P., Hakiza, G., Kalonji, A., Tshilenge, P., Janzac, P.,Roussel, V. Maraite, H. and Rutherford, M.. Coffee Wilt Disease: A Major Constraint To Coffee Production In Africa. European Fusarium Seminar 19-22 september 2006. Wageningen. The Netherlands

CONCLUSIONS

This project provided the first opportunity for scientists from 5 countries to work together and pool their skills to control a major threat to robusta coffee growing in Africa. The complementary approaches taken are a key to the success of such projects which can only be undertaken in their entirety by pooling the skills of North and south countries. This complementarity is particularly illustrated by the results obtained from the analysis of host/pathogen interactions, which could only be undertaken outside a coffee producing zone. Likewise, the transfer of genetic resources between partners in the developing countries could only be envisaged by setting up a quarantine zone.

Several direct outcomes can be attributed to the major results of the project:

- Identification of 4 groups of strains making up the *Gibberella xylarioides* Complex responsible for coffee wilt disease on its first occurrence and on its re-emergence.
- Enrichment of the *Coffea canephora* collections following surveys in the primary forests of Uganda and identification of a new *C. canephora* genetic group which constitutes a new reservoir of genes available for variety creation.
- Identification of planting material that is resistant to coffee wilt disease currently undergoing multiplication to study its performance under different agroecological conditions, and particularly two elite clones in Uganda.
- Proposal for a resistant coffee tree selection scheme.
- Proposal for a rational scheme to limit the spread of the disease in newly infected plots.
- Training of several researchers from North and South countries.

The project has provided new knowledges for a major re-emergent disease. It revealed the need to continue collaboration between countries, as this disease is continuing to spread in Africa. It also

highlights the major risk of an erosion of *Coffea canephora* and *C. arabica* genetic resources unless drastic conservation measures are rapidly taken. Indeed, as this disease kills trees, it also leads to the disappearance of susceptible parents which are often high-yielders. *In situ* conservation measures outside affected zones should be undertaken. The work completed sheds some scientific light for requesting calls for the installation of a conservation centre and backing requests that might be made to research organizations or international funding agencies.

Once again, it is clear that the multidisciplinary approach between the different partners made it possible to clearly identify the problem, propose solutions and provide some concrete achievements, enabling an effective contribution to solving the problem.

The outputs generated by the project activities have already contributed to, and accelerated, the search for a solution to CWD as a devastating constraint to current coffee production. They also provide a sound basis on which future research and development efforts to tackle this and related diseases may be built, specifically with regard to our increased capacity to identify and monitor the differing forms of the pathogen and to identify, develop and deploy coffee with suitable host resistance. It will therefore be of immense importance in supporting and sttrenghtening the breeding programmes already active in affected regions of East and Central Africa.

Scientifics subjects – Future actions

Identify new resistant clones
In-depth analysis of the heritability of resistance
Identify the primary focus of the disease
Check the resistance of resistant material under different agroecological conditions
Analyse the genetic diversity of the available germplasm in DRC
Contribute to developing an international preservation centre outside an infected region

INDIVIDUAL FINAL REPORT

PARTNER P1: Centre de Coopération Internationale en Recherche Agronomiquepour le développement (CIRAD)
France

Daniel BIEYSSE

1- Objectives

CIRAD, the coordinator of the INCO project between CAB International (CABI, Egham, UK), Centre de coopération internationale en recherche agronomique pour le développement (CIRAD, Montpellier, France), the University of Kinshasa (UNIKIN, Kinshasa, Democratic Republic of Congo - DRC), the Coffee Research Institute (CORI, Kituza, Uganda) and the Catholic University of Louvain (UCL, Louvain-la-Neuve, Belgium) provided scientific leadership for the COWIDI project from 1/11/2001 to 31/10/2006 and organized different workshops attended by all the partners, or just some of them depending on the circumstances.

The general aim of the project was to control Coffee Wilt disease of the *canephora* coffee tree, whose causal agent is *Fusarium xylarioides* Steyaert, the perfect form of which is *Giberella xylarioides* Heim. and Saccas. It was reported for the first time in 1927, in the Central African Republic, on *Coffea liberica* var. *dewevrei,* where, as a consequence, cultivation of that species disappeared between 1930 and 1950. It was then reported in Ivory Coast, leading to the disappearance of local *C. canephora* species, and in Guinea. In Cameroon, it led to the disappearance of the species *C. liberica* in the 1950s. It has been reported in DRC since 1947, where it destroys *C. canephora* plantations.

Operations to up root and burn diseased trees, and the dissemination of resistant varieties, resulted in the eradication of the disease at the beginning of the 1960s.

In 1957, the disease was reported for the first time on C. *arabica* in Ethiopia and has never been eradicated.

It was reported again in the mid-1980s in North Kivu province on *C. canephora* in DRC, then spread in all directions, mostly following roads. It was reported for the first time in Uganda in 1993, then in Tanzania in 1997. It has not reappeared in Ivory Coast, Guinea or Cameroon.

This re-emerging disease is a serious threat to all *C. canephora* and *C. arabica* producing countries.

Up rooting and burning infected coffee trees proved to be unfeasible on such a scale and the commercial varieties available are susceptible to the disease. Replanting on infected soil is very hazardous as the pathogen remains in the soil for many years.

The search for resistant varieties proves to be the only strategy for controlling this disease in a sustainable manner adapted to an agro-economic context of smallholders.

CIRAD's more specific aims, in relation with the 4 partners, were:

1- leadership and scientific and administrative coordination,

2- a structural analysis of pathogen populations,

3- an analyze of plant/pathogen interactions,

4- identification of sources of resistance

5- student and researcher training,

6- plant material transfer.

2- Activities

2-1- Organization of workshops

Scientific leadership and coordination of activities was provided during different workshops, attended by all or some of the partners depending on the opportunities for getting together.

2-2- Development of a *Fusarium xylarioides* collection

A collection of *Fusarium xylarioides* isolates responsible for Coffee Wilt was established. It was intended for studying the population structure of the pathogen, characterizing host/pathogen interactions and screening varieties. The samples were collected from throughout the Ugandan territory and in the main infested zones in RDC. In addition, isolates preserved in international fungus collections dating from the first emergence of the disease were ordered. Isolates from Ethiopia supplied by CABI and EARO, collected from *C. arabica*, completed the collection.

The latest introductions came from samples of coffee trees collected in the primary forests of Itwara and Kibale in Uganda.

The isolates from Uganda and DRC were isolated and cloned from fragments of infected stems.

2-3- Implementation of the population structure study using microsatellite markers

A collection was established containing 150 strains gathered from all the geographical zones affected by Coffee Wilt (DRC, Uganda, Tanzania, Ethiopia) and from different coffee species (*C. canephora, C. arabica, C. excelsa*). Historical strains kept in the International Collection collected before the re-emergence of the disease were also studied.

A microsatellite-enriched library was prepared using reference strains of *F. xylarioides* (*CAB 003*) from CAB International (UK) according to the method described by Billote & al. (1999).

The data analysis of 48 sequences was used to designate 11 microsatellite primer pairs. PCR amplifications for each of the 11 microsatellite loci were performed for all the isolates using the designated primer pairs.

The different alleles obtained at the loci considered were converted into a numerical matrix. A dissimilarity matrix was calculated according to the Sokal-Michener index: D(i,j)=u / (m+u) ("u"= the number of identical characters between individuals "i" and "j", and "m"= the number of different characters between the same individuals). The DARWIN 4.0 program (CIRAD, France) was used to calculate a distance matrix and plot a dendrogram by the "weighted Neighbour-Joining" method. To determine the robustness of the dendrogram, the data underwent bootstrap resampling with 100 removals and replacements.

An estimation of genetic variability, "H", within the *F. xylarioides* population was calculated using the method described by W-H. Li and D.Graur (1991).

2-4- Development of a pre-selection test

Development of an inoculation technique to reproduce the infection cycle of the fungus on a plant was necessary for analysing the biological cycle of the pathogen, characterizing resistance and screening planting material.
Two techniques were developed, firstly inoculation using a syringe to inject a calibrated solution of 2.10^6 conidia/ml into the stem of 8 to 12-month-old plants, and secondly by wounding 10 to 12-week-old coffee seedlings with a scalpel dipped in a calibrated conidia suspension. The inoculated coffee plants were then placed in a climatic chamber at 25°C with a photoperiod of 12 h/12 h.

2-5- Evaluation of isolate aggressiveness

Characterization of resistance required precise knowledge of the aggressiveness of the strains studied, and reference isolates had to be defined which were then routinely used to study interactions and assess planting material resistance. To do that, different isolates from Uganda and DRC were inoculated using the standard inoculation programme on a susceptible progeny of *C. canephora*.

2-6- Characterization of *Gibberella xylarioides* complex / *Coffea* spp. interactions

As Coffee Wilt exists on several species, *Coffea canephora, C. arabica, C. liberica*, it was necessary to analyse host/pathogen interactions with isolates isolated from the three species and check their host spectrum. Such an approach is essential in a selection programme for durable resistance. To achieve that, a range of isolates was tested on the three species of *Coffea*. Seeds were provided by different partners or research centres, IRAD (Cameroon), EARO (Ethiopia), CATIE (Costa Rica), SNRA Ivory Coast.

2-7- Screening of genetic resources for resistance to CWD.

The search for resistant coffee trees required the assessment of different genetic resources. They consisted either of material being selected in performance trials, or genetic resources from collections. Different suppliers provided seeds:
- Uganda, CORI: material undergoing selection
- DRC, UNIKIN: material undergoing selection
- CIRAD French Guiana: material in a collection representative of the different known genetic groups of *C. canephora*, *C. liberica* and *C. dewevrei*, for interaction studies.
- Costa Rica (CATIE): *C. liberica* and *C. arabica* for interaction studies,
- Ivory Coast: wild planting material
- Ethiopia: 2 *C. arabica* lines.

2-8- Researcher and training supervision

Training operations were scheduled under this project, both with North and South partners. The trainees were supervised by the UMR BGPI plant pathology laboratory and the plant pathologists, geneticists and biometricians associated with the project.

Funding was provided by the project, but also from CIRAD's own resources.

2-9- Establishing MTAs.

The transfer of genetic resources between partner countries was made possible by the signing of MTAs setting out the conditions for plant material transfers between Uganda and CIRAD on the one hand, and between Ivory Coast, CIRAD and Uganda on the other hand.

3- Results

3-1- Organization of workshops

Workshops and meetings by way of hosting researchers from the South at CIRAD or UCL were regularly staggered throughout the duration of the project.

4/03/2002 – 7/03/2002. This first workshop was attended by all the partners and took place in Kampala to take stock of our knowledge and lay the foundations for the activities and objectives of each team.

26-28/02/2003. UCL visited CIRAD laboratories to discuss inoculation techniques and first results.

5-10/06/2003. Pr Kalonji visited UCL and CIRAD to discuss the first results and draw up Tshilenge's work programme.

13/10/2003 – 17/10/2003. CIRAD visit to CORI. Coordination of activities proposed at the initial workshop and, more particularly, launching a work programme as part of Mr. Pascal Musoli's thesis.

18-19/11/2003. Workshop with at least one participant from every partner, except CORI, at UCL. Presentation of results concerning genetic diversity of the fungus, sexual cycle and host/pathogen interaction. Drawing up of the next work programme.

12/2003. Rutherford visited CORI to discuss activities relating to epidemiological studies, analysis of data from field trials.

29/06/2004. Workshop with at least one participant from every partner, except UNIKIN, at UCL. Presentation of results concerning genetic diversity of the fungus and Ugandan *Coffea canephora*, mating type and host/pathogen interaction. Drawing up of the next work programme.

23/11 -4/12/2004. Bieysse, visited CORI in connection with surveying wild coffee trees and to discuss activities relating to epidemiological studies, analysis of data from field trials.

27/01 – 07/02/2005. Bieysse, Leroy visited CORI in connection with surveying wild coffee trees and to discuss activities relating to epidemiological studies, analysis of data from field trials

13/09/2006. Final workshop in Montpellier with all the participants.

During this project there were many exchanges via researchers from CORI and UNIKIN receiving DEA and thesis training at UCL and CIRAD.

3-2 –Development of a *Fusarium xylarioides* collection

A collection of 150 monospore isolates was established from fragments of wilt infected wood. Those isolates were collected during surveys in Uganda and DRC. They consisted of *Fusarium xylarioides* and also saprophyte species present on the wilted pieces of wood. Most of them have undergone mycological description and assessment.

Some isolates were received from isolations carried out at UCL or CABI.

That basic collection has been enriched with various samples from countries not taking part in the project, such as Tanzania, a country newly affected by the epidemic, and Ethiopia (EARO), associated through outside collaboration on the study of this disease present on *C. arabica*.

This set makes up the basics of the contemporary isolate collection corresponding to re-emergence of the disease.

Another part of the collection comes from samples collected during the first spread of the disease and conserved in international fungus collections. They correspond to the "historical isolates".

Under this project, there were numerous exchanges of strains between the partners.

3-3- Implementation of the population structure study using microsatellite markers

The molecular markers used revealed low genetic variability in the species *F. xylarioides* H = 0.10. The genetic diversity found within the species *F. xylarioides* was mainly due to the historical strains and differentiation between the isolates versus *C. canephora* and versus *C. arabica*.

Isolates were collected from a maximum number of geographical zones affected by Coffee Wilt, in order to ensure the best possible representation of geographical diversity within the set of isolates studied. However, there were no correlations between the geographical distribution of the isolates and molecular diversity. The dendrogram (fig.1) shows perfect homogeneity between the isolates collected in Uganda, the Democratic Republic of Congo and Tanzania (group ***A***). Likewise, in group ***B*** (fig.1) no genetic variability (H=0) could be found between the isolates collected in Ethiopia from *C. arabica*.

However, there was a degree of genetic diversity between the isolates collected from the species *C. arabica* and those collected from the species *C. canephora* and *C. excelsa*. Those results back up the hypothesis of host specificity reported during field observations of *F. xylarioides* in relation to the species *C. arabica* (Van Der Graaff et al. 1977; Pieters et al. 1978) and recent observations in DRC in the Mutwanga region where plantations are composed of both *C. canephora* and *C. arabica*, but where only *C. canephora* plants are attacked.
However, the historical strains (*CBS 258.52* and *749.79*, *ATCC 156.64* and *DsMZ 624.57*) revealed a degree of genetic variability within the species *F. xylarioides*. These strains were collected from coffee trees of the *C. canephora* and *C. excelsa* types in the 1950s-1960s during the first wave of infections and differed between *C. canephora*/*C. excelsa* and *C. arabica*. These historical strains also displayed diversity between themselves. The *CBS* strains collected in West Africa (Guinea, Ivory Coast) differed from strain *DsMZ 624.57* collected in the Central African Republic. Current data is insufficient to reach any conclusion as to the origin of the diversity observed, or to possible evolution of the species. The hypothesis of selection pressure generated by the massive eradication of the disease in the 1950s and the use of resistant *C. canephora var. robusta* clones may explain the low diversity observed within current strains, whereas diversity does exist within historical strains.
The low genetic diversity found within the species *F. xylarioides* suggests clonal multiplication of the pathogen since the re-emergence of the disease. However, the teleomorphic form *Gibberella xylarioides* that has been described (Heim, 1954) suggests that it is involved in genetic recombination phenomena. Moreover, perithecium formation in the field is very often seen. Given that these phenomena are part of the mechanisms responsible for the introduction of high genetic diversity within a species, it seems essential to take a closer look at the sexual cycle of *F. xylarioides*.

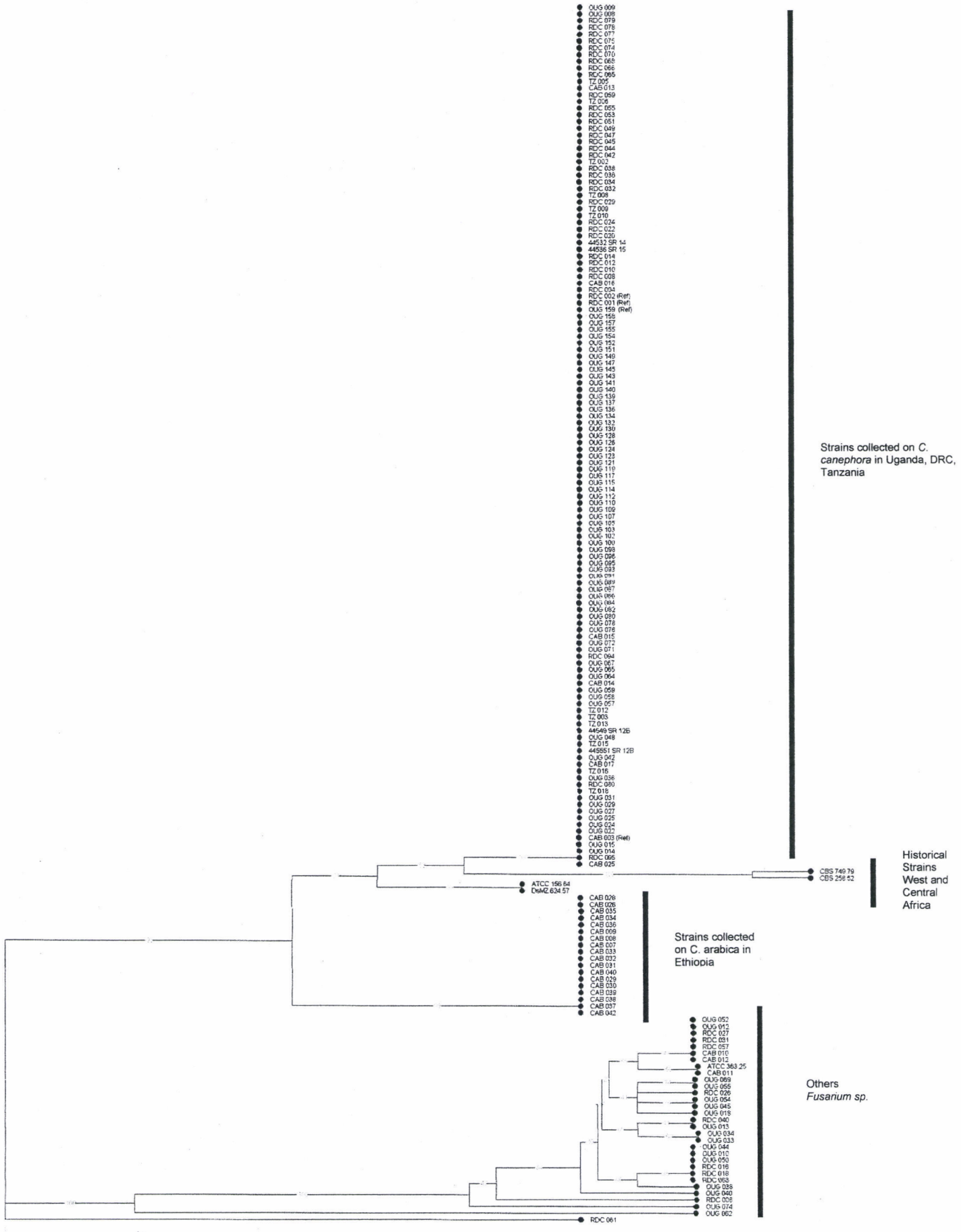


Fig 1. Unrooted Neighbor Joining dendrogram obtain after calculation of distances according to the Sokal-Michener index from the polymorphism observed with 11 microsatellite markers.

3-4- Development of pre-selection tests

Two standardized inoculation methods have been developed.

Their protocol is described in 2-4.

The method on 9 to 12-month-old plants by injecting a calibrated suspension was developed during the first trials. It was reliable and reproducible, but took up greenhouse space and labour over quite a long period. Likewise, it took up more space in the climatic chambers.

Consequently, the inoculation technique by wounding with a scalpel on batches of twenty 10 to 12-week old seedlings was preferred, as it could be implemented more quickly and required less room.

Every trial was validated with a susceptible control inoculated with the reference strain CAB003. The results were analysed with a statistical design based on the Generalised Linear Model.

3-5- Evaluation of isolate aggressiveness

The aggressiveness of 34 "contemporary" isolates collected from *C. canephora* in DRC, Uganda and Tanzania was assessed. The aggressiveness of the isolates induced mortality rates ranging from 10 to 100%. Wide variability in aggressiveness, expressed in the form of a continuum, was observed. Isolate CAB003 from Uganda was chosen as the reference isolate used for all the tests.

3-6- Characterization of *Gibberella xylarioides* complex / *Coffea* spp interactions

All the isolates collected from the different species of *Coffea* were assessed in crossed inoculations.

Isolate ATCC36325 was not pathogenic. That isolate does not belong to the species *F. xylarioides*. It was wrongfully described by Booth as a female strain. This result confirms the conclusions of P. Lepoint and our own results obtained with microsatellite markers excluding that species.

Isolates CBS74979 from Guinea and CBS25852 from Côte d'Ivoire confirmed their pathogenicity on the species *C. canephora*. They also proved to be pathogenic on the species *C. dewevrei*, which is not the species from which those strains were collected.

Although isolates ATCC15664 and DSMZ62457 were isolated from *C. dewevrei*, they were pathogenic on the two species *C. canephora* and *C. liberica*.

Those historical isolates tested displayed a broad host spectrum, that could induce young plant mortality on both species, *C. canephora* and *C. dewevrei*, and isolate DSMZ62457 was also pathogenic on the 3 species (trials C, E).

Isolate CAB003 was specific to the species *C. canephora*. One exception was found in trial D, where it induced mortality on young *C. dewevrei* plants.

Although these results are only partial. It was quite complicated to proceed a complete crossed inoculation due to the lack of *C. liberica* seeds in sufficient quantities. They nonetheless show that the historical isolates had a broad host spectrum than the contemporary population represented by isolate CAB003.

Isolate CAB007 seemed specific to the species *C. arabica*, despite the 5% mortality obtained with a progeny on *C. dewevrei* and *C. canephora*. The specificity of isolate CAB007 to the species *C. arabica* therefore seems to have been confirmed in the growing zones where both species exist side by side. In Ethiopia in the Jima zone, at the EARO research centre in a severely contaminated area, only *C. arabica* plants were affected, with *C. canephora* plants remaining unaffected.

The interactions are summarized up in table 1.

	CAB003	CAB007	DSMZ62457	CBS25852	CBS74979	ATCC15664	ATCC36325
C. canephora	+++	-	+	+	+	+	-
C. liberica	+		+++	+	+	+	-
C. arabica	-	++	++				-

3-7- Screening of genetic resources for their resistance to CWD.

All the inoculations were carried out with isolate CAB003. The results (Table 2) corresponded to the percentage of dead plants obtained a minimum of 100 days after inoculation. The replicate number is indicated. The evaluation corresponded to seeds collected from one individual plant identified in a plot.

Coffee trees displaying infected plant rates below 10% were considered in the first analysis to be tolerant and possibly a reservoir of potentially usable genes of resistance for a breeding programme. Those results needed to be confirmed by a new series of artificial inoculations and verification in the field in an infested zone.

The resistance of the coffee trees belonging to the Guinean group was confirmed. Tested coffee trees from that group displayed infected plant percentages ranging from 0 to 5%. However, a few Guinean accessions exceeded that percentage.

The progenies with a mortality rate below 20% belonged to the different genetic groups of the species *C. canephora*, apart from group C, which was not represented. It would seem that the different genetic groups whose progenies had mortality rates over 20% were randomly distributed.

A general analysis of the results seemed to indicate a decrease in the susceptibility of progenies in line with plant age. That tendency was observed in several replicates with progenies 1162, 1167, 1173, 1186, 1331, 1333, 1366, 1375, 1377, and tissue lignification mechanisms may have played a part in plant defence mechanisms. Changes in the susceptibility of plants depending on their age needed to be taken into account when characterizing planting material resistance, specifying the age of the inoculated plants (data not shown).

It was not possible from these exploratory results to identify a genetic group that was more likely to bear resistance factors. A few resistant progenies were found in all the groups.

Moreover, progenies found to have a mortality rate of 0% or under 5% will have to be confirmed and will be selected as a source of resistance to Coffee Wilt.

The DRC coffee populations, with infected plant rates of under 10%, and particularly KR19/4, S19/29, TR-CI-22/21 and LK35/2 with infected plant rates of under 6%, would be worth testing in a naturally infected area.

These results show that sources of resistance that can be detected by early screening. Resistance needs to be confirmed in a natural infection zone before using these genetic resources in a breeding programme. All the results taking into account the mortality percentage are shown in the following table.

Table 2. Mortality percentages for the genetic resources tested with CAB003 (n: replicate number)

DRC			
Population	CIRAD code	Mean	n
KR 19/4	1374	0	1
S 19/29	1644	0	1
TR-CI 22/21	1677	5	1
LK 35/2	1937	6	1
LK 56/1	1939	7	2
LAF 159/20	1632	12	4
KR 12/4	1334	12	3
LK 22/6	1636	12	3
KR 12/4	1373	15	2
S 19/19	1642	17	1
S 19/23	1643	17	1
S 9/42	1641	17	1
TR-CI 17/37	1673	17	2
LAF 159/33	1634	17	2
S 19/58	1647	18	2
LAF 159/58	1635	19	1
TR-CI 18/36	1674	20	2
S 19/69	1648	22	2
LR/R1P2 (7)	1650	22	2
S 19/73	1649	23	2
KR 1/3	1332	25	2
TR-CI 20/40	1676	25	1
LAF 159/23	1633	27	2
S 9/23	1640	27	4
TR-CI 23/34	1678	28	1
KR 2/5	1333	28	5
LR/R3P2 (3) SO	1656	30	2
KR 16/5	1335	30	3
KR 19/12	1336	30	3
TR-CI 14/1	1666	30	1
KR B/11a	1375	31	4
S 19/46	1645	33	1
TR-CI 15/11	1667	33	1
TR-CI 24/30	1680	33	1
TR-CI 17/33	1672	38	2
S 19/52	1646	39	1
KR B1/1	1339	40	1
TR-CI 1/20	1657	40	3
LR/R1P3 (17)	1653	41	2
TR-G 15/13	1684	42	2
TR-CI 12/24	1665	43	1
LR/R2P4 (14)	1654	44	3
TR-CI 17/17	1671	44	1
KR 1/3	1370	45	1
TR-CI 10/5	1663	45	1
TR-CI 7/4	1660	45	2
TR-CI 5/21	1659	46	2
LR/R2P4 (25)	1655	46	3
KR A/1	1337	47	3
TR-CI 1/25	1658	50	2
L 251	1924	50	1
LR/R1P2 (16)	1652	50	2
L 147	1923	55	1
LR/R1P2 (11)	1651	55	2
SA 158	1925	55	1
TR-CI 24/35	1681	55	1
KR B/14	1377	56	4
KR B/14	1341	63	3
TR-CI 11/23	1664	75	1
TR-CI 23/39	1679	75	1
KR B/11b	1376	85	1

Guinean genetic resources			
Genetic group	CIRAD code	Mean	n
Guinean	1174	0	1
Guinean	1175	0	2
Guinean	1422	0	1
Guinean	1423	0	1
Guinean	1424	0	1
Kouilou	1425	0	1
Guinean	1434	0	1
Guinean	1435	0	1
Guinean	1436	0	1
Guinean	1437	0	1
Guinean	1438	0	1
Guinean	1439	0	1
Guinean	1440	0	1
Guinean	1173	2	3
Congolese	1344	5	2
Kouilou	1433	5	1
Kouilou	1168	6	1
Guinean	1172	6	1
Guinean	1350	6	5
202	1409	10	1
"nemaya"	1607	11	5
Nana	1342	12	2
Congolese	1416	15	1
HA 469	1606	15	1
Guinean	1351	20	1
Nana	1159	27	1
Congolese	1161	29	2
200	1181	29	3
200	1360	30	2
503	1369	30	1
197	1179	31	1
200	1361	35	2
Congolese	1162	37	3
197	1358	38	2
C. canephora	1331	38	9
Congolese	1419	40	1
Congolese	1420	40	1
Kouilou	1169	42	2
200	1180	42	1
197	1359	43	4
Nana	1343	43	3
200	1362	45	1
C.canephora	1151	47	4
400	1187	47	2
197	1177	48	3
Nana	1157	49	1
Congolese	1163	50	1
Congolese	1164	52	3
202	1185	52	2
Kouilou	1167	52	2
400	1366	53	5
197	1357	53	3
197	1178	54	2
400	1368	58	3
400	1186	61	2
119	1143	62	1
Kouilou	1170	64	1
Nana	1158	65	2
202	1184	72	2
202	1407	80	1
Kouilou	1430	80	1
400	1188	92	1

IVORY COAST				
Genetic group	Population	CIRAD code	Mean	n
Guinean	2183	1224	0	2
SG2	2651	1233	5	1
Nana		1342	12	2
SG2	57	1210	12	3
B	2571	1230	18	1
B	2507	1227	20	1
SG1	A 03	1192	22	3
KB	KB 8	1203	24	2
C	Na 090	1197	29	3
C	Na 054	1195	33	1
SG2	392	1218	35	1
SG2	464	1220	37	4
B	2576	1231	38	1
C	Na 089	1198	40	3
KB 1	KB 1	1200	40	1
B	2593	1232	42	1
C	Na 063	1196	43	1
SG2	32	1209	43	1
Nana		1343	43	1
SG2	121	1213	45	1
SG2	69	1211	56	2
SG2	2	1208	60	3

UGANDA Hybrids			
Population	CIRAD code	Mean	n
J 3 1	1903	30	1
Q 3 4	1908	30	2
C 6 1	1899	30	1
B 1 1	1894	32	2
R 1 4	1910	33	2
L 2 7	1904	35	2
B 2 1	1895	37	2
Q 6 1	1909	38	2
P 5 1	1906	42	2
Q 1 1	1907	46	2
B 6 2	1896	46	2
H 4 1	1902	47	2
G 3 7	1901	49	2
E 3 2	1900	49	2
P 3 6	1905	65	2

DRC Germplasm		
Abbreviation	Station	Observation
KR	Kiyaka	
LAK	Luki	Lafarge estate
LK	Luki	Local survey
S	Luki	Local survey
LR	Luki	Transfer from Yangambi
TR-CI	Tshela	Introduction from Ivory Coast
TR-G	Tshela	Introduction from Guatemala

3-8- Supervision of researchers and training.

Implementation of this project enabled the hosting of numerous trainees undergoing university training, or researchers.

Mateo C. Coffee Wilt of *C. canephora* due to *Fusarium xylarioides Steyaer* : Study of host/parasite interactions and trial to develop an early inoculation test. Masters in Applied Plant Physiology. April–September 2001. University of Montpellier II.

Dupuy L. Study on Coffee Wilt of robusta coffee. Validation of an early screening test. Study of interaction. IUP Agro science. April-September 2002. University of Avignon.

Pierre S. ITS analysis of *Fusarium xylarioides*. April–September 2003. University of Montpellier II.

Pierre S. ITS analysis of *Fusarium xylarioides*. April–September 2003. University of Montpellier II.

Villar E. Contribution to the study of Coffee Wilt on Robusta coffee due to *Fusarium xylarioides*. IUP Agro science. April-September 2003. University of Avignon.

Janzac B. Biodiversity study of *Fusarium xylarioides*, a phytopathogenic fungus responsible for Coffee Wilt. DESS Plant Protection and Utilization. April-September 2003. Université de Pau et des pays de l'Adour.

Girma Adugna. Comparative analysis of *Fusarium xylarioides* interactions on two species, *C. canephora* and *C. arabica*. EARO (Ethiopian Agricultural Research Office) Part of thesis work. November 2003-March 2004. University of Bonn.

Musoli Pascal. Search for sources of resistance to Coffee Wilt of *Coffea canephora* Pierre, due to *Fusarium xylarioides* Steyaert in Uganda. Thesis 26-03-2007. University of Montpellier II. France.

3-9- Establishment of MTAs

Several Material Transfer Agreements were signed between the project partners or with associate partners, enabling transfer of the plant material intended for the studies.

Two MTAs were established:
CORI – CIRAD
CATIE – CIRAD

One transfer agreement:
SNRA Ivory Coast – CIRAD

Material transfer with UNIKIN was carried out under the project without any special rider.

4- Problems encountered

No particular problem was encountered during implementation of the work packages. However, unfavourable meteorological conditions in Uganda, *a* long dry period in 2004, resulted in very low pollination rates. Those pollinations had to be repeated in 2005, leading to a request for a 1-year project extension, without any modification to the budget, in order to fulfil the objectives fixed. The 15 deliverables identified at the beginning of the project were achieved

5- Technology implementation plan

The fundamental result of the COWIDI project is a structural analysis of the *Fusarium xylarioides* population and a clearer understanding of *Coffea canephora* resistance, along with the identification of resistance sources in existing genetic resources, or collected over the project period.

No "product" suitable for direct commercial development was produced during the project.

The results obtained have been or will be published and will be accessible to the public.

6- Publications and papers

Oral presentations

Janzac Bérenger, Roussel Véronique, Bonnemaire Katia, Hakiza Georgina, Kalonji Adrien, Pinard Fabrice, **Bieysse Daniel**. Etude de la biodiversité de *Fusarium xylarioides* agent de la trachéomycose des caféiers. Implications dans le schéma de sélection de *Coffea canephora*. 5ème rencontres de Phytopathologie. 18-22 janvier 2004 Aussois France.

Pinard Fabrice, **Bieysse Daniel**, Leroy Thierry, Dufour Magali. Regional Coffee wilt Programme stakeholders and planning Workshop. "Breeding strategy for CWD resistance based upon CWD diversity". Nairobi, Kenya. 9-13 November 2004

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

Pascal Musoli, Pauline Aluka, Philippe Cubry, Magali Dufour, Fabien de Bellis, James Ogwang, Denis Kyetere, Thierry Leroy, **Daniel Bieysse**, André Charrier. Fighting coffee wilt disease: Uganda wild *C. canephora* genetic diversity and its usefulness. ASIC 11-16/09/2006. 21st International Conference on Coffee Science, Montpellier, France

Papers submitted

Pascal Musoli, Philippe Cubry, Pauline Aluka, Claire Billot, Magali Dufour, Fabien De Bellis, Denis Kyetere, James Ochugo, **Daniel Bieysse**, André Charrier and Thierry Leroy. A new genetic diversity group from Uganda within *Coffea canephora* Pierre. Submitted to the Genetics Journal

Pascal C. Musoli, Christian Cilas, David Pot, Agnes Nabaggala, Saleh Nakendo, James Pande, André Charrier, **Daniel Bieysse**, Thierry Leroy. Inheritance of resistance to coffee wilt disease *(Fusarium xylarioides* Steyaert) in *Coffea canephora* Pierre. Submitted to New phytologist Journal

Pascal C. Musoli, C. Cilas, A. Charrier, A. Kangire, G.M. ten Hoopen, C. Kabole, J. Ogwang[1] and **D. Bieysse**. Spatial and temporal analysis of Coffee Wilt Disease caused by *Fusarium xylarioides* Steyaert in *Coffea canephora.* Submitted to European Journal of Phytopathology

Participation to congress

JANZAC Bérenger, MUSOLI Pascal, ROUSSEL Véronique, BONNEMAYRE Katia, PINARD Fabrice, LEROY Thierry, DUFOUR Magali, KYETERE Denis, HAKIZA Georgina, TSHILENGE Patrick, KALONJI Adrien, GIRMA Adugna, **BIEYSSE Daniel**. Control of Coffee Wilt: study of genetic diversity *Fusarium xylarioides* and *Coffea canephora* in Uganda. 20th International conference on Coffee Science. 11-15 Octobre 2004 Bangalore India.

Bieysse, D., Lepoint, P., Hakiza, G., Kalonji, A., Tshilenge, P., Janzac, P.,Roussel, V. Maraite, H. and Rutherford, M.. Coffee Wilt Disease: A Major Constraint To Coffee Production In Africa. European Fusarium Seminar 19-22 september 2006. Wageningen. The Netherlands

7 Conclusions

Application of microsatellites markers revealed limited genetic diversity within the fungus, suggesting little genetic evolution of the species and predominance of vegetative propagation, despite observation of a sexual stage (perithecium formation) in nature. Results highlighted two major genetic groups that correlate directly with the *Coffea* species from which strains were obtained, i.e. *C. canephora/C. liberica* and *C. arabica*.

Furthermore, strains obtained from *C. arabica* and *C. canephora* induced CWD symptoms only on their respective host species when challenged against coffee seedlings of a range of differing species, suggesting show strict host specificity. However, inoculation with strain DSMZ 62457, obtained from *C. excelsa* in CAR, induced symptoms not only on *C. excelsa* but also on *C. canephora* and *C. arabica*. This suggests that some strains may display a larger host range encompassing not only one but several cultivated *Coffea* species.

The findings of this work are of fundamental importance to the future management of CWD and in safeguarding the livelihoods of millions of smallholder coffee farmers across the African continent.

Complementarities between the partners were very productive and strong. Findings, have allowed development of standardised artificial inoculation, new knoledge on genetic diversity of *F. xylarioides,* identification of resistant clones and identification of a new pool of genes available for variety creation.

INDIVIDUAL FINAL REPORT

PARTNER P2: Université catholique de Louvain (UCL) Belgique

Dr Pascale Lepoint
Pr Henri Maraite

1. OBJECTIVES

The **COWIDI** (Coffee Wilt Disease) **project**, funded by the European Union, was created in the framework of an international scientific cooperation project (INCO) in order to implement a long-term strategy based on durable resistance against coffee wilt in Africa. The global aim of this collaborative research program between CAB International (CABI, Egham, UK), Centre de coopération internationale en recherche agronomique pour le développement (CIRAD, Montpellier, France), the Université de Kinshasa (UNIKIN, Kinshasa, Democratic Republic of Congo - DRC), the Coffee Research Institute (CORI, Kituza, Uganda) and the Université catholique de Louvain (UCL, Louvain-la-Neuve, Belgium) was to improve the knowledge of the coffee wilt pathogen, *Gibberella xylarioides*, and determine its potential diversity at a morphological, biological, genetic and pathogenic level.

On a more specific level, **UCL** was implicated along with CABI and CIRAD in the characterization of the diversity of the pathogen at a mycological, pathogenic and molecular level using a range of techniques to determine whether a new strain of the pathogen had surfaced and become dominant. UCL investigated diversity at a specific level using morphological, biological (sexual compatibility) and molecular tools (RAPD, elongation factor, calmodulin, histone 3, mating type). As agreed at the First General Meeting of the project in Kampala Uganda, UCL equally tried to focus on the mechanisms of variation of the coffee wilt pathogen through the sexual and asexual cycle, in close cooperation with CORI and UNIKIN, involved among others in studying the life cycle and the pathogen-coffee genotype interactions *in situ*. This information is crucial to help understand why the disease has re-emerged, and if the re-emergence is due to a new, more aggressive strain, or to a new taxon. Reference cultures have been stored for long-term conservation on SNA slants, in lyophilised form and by cryopreservation (-130°C) in BCCM™/MUCL facilities.

In the scope of the COWIDI project, a 43-month PhD grant within the 5-year period of the project was allocated on November 1st 2002 to Pascale Lepoint (MS in agronomy) whom obtained her PhD degree on December 21st 2006. UCL also assisted UNIKIN in the characterization of fungi associated with coffee wilt symptoms in the DRC by cross-checking isolates from samples collected in the field and providing a 12-month mycological training course (DEA) to UNIKIN scientist Ir. Patrick Tshilenge-Djim.

2. ACTIVITIES

2-1. Establishment of a collection of coffee wilt strains

A collection of *Fusarium* spp. strains associated with coffee wilt symptoms was established in a first step towards the understanding of Coffee Wilt Disease (CWD), its lifecycle and the possible reasons for its re-emergence in the Congo Basin region. Congolese wilt-stricken Oriental (Isiro and Yangambi, 2002), North Kivu (Beni and Butembo, 2002) and Equatorial (2004) Provinces were surveyed and sampled by UNIKIN staff. 20-30 cm long stem samples were removed from the collar region of sick trees and sent off to CIRAD and UCL labs for isolation.

Isolations were performed at UCL in duplicates from aseptic bark regions and from internal tissues. Stem samples were surface sterilized before being split length-wise, through the revealed wood staining or perithecia. Pieces of ~1 mm² were removed from the different zonations observed in the wood throughout the transect, placed onto 2% water agar (WA) and incubated at 25°C in the dark. When sufficient growth was observed, cultures were purified by transferring ~4 mm² pieces of culture from actively growing margins of the colony to SNA plates and then purified by single-spore isolation.

The collection was completed with strains of diverse geographical and *Coffea* spp. hosts obtained from CABI and CIRAD partners as well as from international culture collections.

2-2. Pathogenicity tests

Inoculations under controlled conditions of various *Coffea* spp. "population-varieties" were carried out in UCL glasshouse facilities using both the stem injection and the scalpel wound technique.

2-3. Diversity Assessment

With the *G. xylarioides* collection well established, the next step was to gain better knowledge of the pathogen. The morphological diversity of strains within the collection was evaluated based on the phenotype of cultures on PDA, the growth-temperature response of strains and the morphological analysis of the teleomorph (produced in artificial crosses).

The first issue to clear was Booth's 1971 statement that *G. xylarioides* is a "*heterothallic fungus with sex-linked morphological characteristics*". To determine the veracity of this statement, it was essential to successfully induce the sexual state *in vitro* using single spore cultures of known origin. The teleomorph had previously been produced *in vitro*; however, the exact conditions, strains and methods used were not described. A second concern that needed to be settled was the origin of the re-emergence of CW in the DRC, and therefore, the subsequent cases declared in Uganda and Tanzania. Are they due to a same pathogen that has spread out from its initial Congolese foci, or are they distinct pathogens?

To help us answer these questions, a crossing assay using carrot agar (Klittich and Leslie, 1988) was applied to *G. xylarioides* strains and associated to their molecular characterization (Lepoint *et al.*, 2005). Conserved regions of the mating type genes (*MAT*) were successfully amplified by *MAT*-PCR using previously described primer pairs (Arie *et al.*, 1999; Kerényi *et al.*, 1999; Steenkamp *et al.*, 2000) designed for *F. oxysporum* and the members of the *Gibberella fujikuroi* species complex (GFC). A global picture of the *G. xylarioides* population was obtained using Random Amplified Polymorphic DNA (RAPD). The molecular diversity was then evaluated by the sequencing of nuclear genes involved in either housekeeping or in mating functions.

3. RESULTS ACHIEVED

3-1. Establishment of a collection of coffee wilt strains

In total, more than 160 *G. xylarioides* hyphal tip isolates and 57 monoascospores were obtained from 40 Congolese *C. canephora* stem samples. Other Fusaria such as *F. solani*, *F. lateritium/stilboides*, *F. oxysporum*, *F. decemcellulare* and at least two other *Fusarium* spp. were equally isolated from symptomatic trees. These saprophytic Fusaria were mostly present in the bark or outer wood regions, and on some occasions in association with *G. xylarioides*. However, two samples from Yangambi yielded only *F. solani* that was capable, like *G. xylarioides*, of pith colonization. A sample from Butembo yielded only *F. stilboides/F. lateritium* in the inner and outerwood regions, while three samples from Butembo and one from Beni yielded numerous *Fusarium* spp. but no *G. xylarioides*.

In conclusion, several Fusaria can be isolated in association with *G. xylarioides* from samples affected by so-called "coffee wilt". Moreover, it is possible that samples can be exempt of *G. xylarioides* or even any *Fusarium* spp. despite observations of symptoms *in situ*. This highlights the difficulty, even for trained staff, to correctly identify coffee wilt symptoms *in situ*. Based on our isolations, *G. xylarioides* is not only associated with a blue-black staining of the wood, but can also be isolated from tissues presenting a grayish, grayish-brown, brown, light-brown, whitish or purplish pigmentation of the wood. *G. xylarioides* was even isolated from healthy looking tissue neighboring stained ones. However, wood staining is not distinctive of *G. xylarioides* since all of the *Fusarium* spp. described here above can be equally associated with these zonations.

With these facts in mind, the hypothesis that infection by several or a combination of *Fusarium* can lead to wilt appears plausible (Tshilenge *et al.*, 2004); however their action as a complex still needs to be assessed.

Twenty *G. xylarioides* strains (assigned number MUCL 47039 to 47058) isolated from Congolese Equator Province CW stem samples collected north of the Congo River in a survey carried out in 2004 were forwarded to UCL for further characterization. Equator strains received were added to reference Congolese *G. xylarioides* strains MUCL 14186 and MUCL 35223 previously collected and identified by MUCL staff, as well as *C. canephora* strains from Uganda collected and isolated by Professor H. Maraite in 2002. The collection was completed with *G. xylarioides C. canephora* strains from Uganda and Tanzania donated by CIRAD and CABI facilities in the framework of the COWIDI project. In order to evaluate the global diversity of the pathogen, Ethiopian *C. arabica* strains and strains isolated in the 1950s to 1960s from *C. canephora*, *C. excelsa* or *Coffea* spp. in the Central African Republic (CAR), Guinea (Conakry) and Côte d'Ivoire were obtained from international culture collections and added to our collection.

All reference strains used in UCL publications will be deposited in the BCCM/MUCL™ international culture collection before 31/07/2007.

3-2. Pathogenicity tests

Despite the fact that typical CWD leaf wilting symptoms were observed, interpretation of results was difficult given that non-inoculated plantlets manifested identical symptoms. Isolations on petioles of fallen leaves revealed the presence of *G. xylarioides*. No mature perithecia were observed in either of the inoculation trials, only perithecial initials could be observed around the collar region of dead seedlings. Exceptionally, strain **IMI 204746** (*G. xylarioides* on *Coffea arabica* from Ethiopia) induced typical leaf wilting symptoms on a *Coffea canephora* plantlet.

3-3. Diversity assessment

3-3.1. Biological diversity

The teleomorph (**Fig 1**) was successfully produced *in vitro* when two morphologically identical strains of opposite mating types were crossed, but not when a strain was "selfed" (crossed with itself).

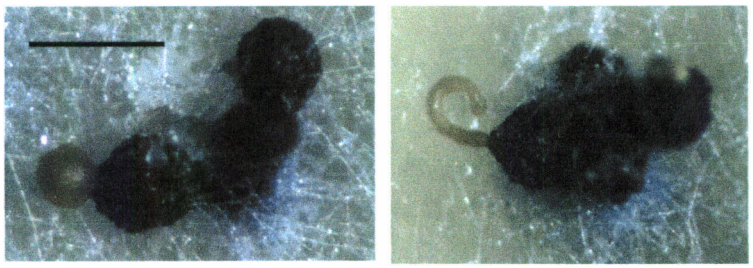


Fig 1 Purple-black *G. xylarioides sensu lato* perithecia producing an orange cirrhus of ascospores on a carrot agar plate when *C. canephora*-associated strains of opposite mating types are crossed (Scale bar, 500 µm).

Sexual compatibility assays between *G. xylarioides sensu lato* strains of divers geographical and *Coffea* spp. origins enabled the identification of three distinct biological species in addition to a residual sterility group containing strains that are incompatible with each other as well as with defined BS (**Table 1**). Reproductive success of each cross was evaluated and scored on a scale with five categories (0 to 4), with 4 representing a cross producing viable progeny and thus assigning the implicated strains to a same biological species (BS) accordingly to Mayr (1940).

Phylogenetic complex			*G. xylarioides* complex			
Host			Ca	Cc	Ce	Cc
Region			Eth	DRC	CAR	Gui, CI,CAR
		BS/SG	BS 1	BS 2	BS 3	SG 4
G. xylarioides complex	Ca	Eth	BS 1	4	2-3	1	1-2
	Cc	DRC	BS 2		4	1-2	0
	Ce	CAR	BS 3			4	0
	Cc	Gui, CI, CAR	SG 4				0

Table 1 Simplified matrix displaying the reproductive success of crosses within and between strains belonging to the *G. xylarioides* complex. Headings for rows and columns indicate the phylogenetic complex designation, host (Ca, *Coffea arabica*; Cc, *C. canephora*; Ce, *C. excelsa*), geographic origin of the strains (Eth, Ethiopia; DRC, Democratic Republic of Congo; CAR, Central African Republic; Gui, Guinea [Conakry]; CI, Côte d'Ivoire), and biological species (BS) and sterility groups (SG) identified in crosses. Numbers within the matrix cells represent reproductive success ratings between corresponding mating type compatible strains with 0 indicating a sterile cross, 1-3 partially fertile crosses and 4 fully fertile crosses producing viable ascospores. A reproductive success of 4 was used to delineate biological species (BS), which are shaded in dark gray.

BS 1 contains Ethiopian *C. arabica*-associated strains, BS 2 includes *C. canephora*-associated strains from the Congo Basin region where as BS 3 encompasses alleged Central African *C. excelsa*-associated strains BBA 62457 and ATCC 15664. Central and West African strains BBA 62455, CBS 25852, CBS 74979 and MNHN 709 appear to be female sterile (except BBA 62455) although still sporulating more or less abundantly. Provided that they belong to the same biological species, they should theoretically be able to interact at least as a male parent and form the perfect state when crossed with compatible female fertile strains such as those defined in BS 1, BS 2 or BS 3. Seeing as no fertile perithecia were formed in crosses implicating these strains, they have been conservatively assigned to a single sterility group, SG 4, on the basis that they originate from a same host and region. Moreover, strains belonging to BS 1 and especially BS 3 showed lower levels of female fertility in intra-BS crosses compared to BS 2. 29 monoascospores were isolated from three different crosses implicating strains belonging to BS 2.

Until recently, the teleomorph had never been formed in carrot agar crosses when compatible strains from the first epidemic (1950s) isolated from *Coffea* spp. in the Central African Republic (CAR), Guinea and Côte d'Ivoire were implicated in a cross. It was believed that fertility had been greatly reduced, if not totally lost, through sub-optimal conservation conditions since initial isolation more than half a century ago. However, strain **BBA 62457 (*C. excelsa*, CAR?, 1971, *MAT-2*)** constitutes an exception since this strain readily produces protoperithecia in individual cultures in addition to crosses, suggesting that its "female fertile" character (i.e., ability to produce perithecial initials) is still intact. The perfect state was obtained recently for the first time in a cross between ATCC 15664 **(*Coffea* sp., Guinea?, 1964?, *MAT-1*)** and BBA 62457 from which 10 ascospores have been isolated. Seeing as these two strains are reproductively isolated from strains belonging to the BS 1 and BS 2, ATCC 15664 and BBA 62457 have been assigned to a distinct biological species (**BS 3**).

Out of the ten viable progeny isolated from the **ATCC 15664 x BBA 62457** cross, two appear to be highly fertile and produce abundant stroma in culture similarly to female parent strain BBA 62457. The other eight strains sporulate to a lesser extent, do not form stromatic structures on PDA, and produce a pinkish pigment in the agar and aerial mycelium just as male parent ATCC 15664. Progeny strains were backcrossed with both parents for *MAT* identification, determination of fertility levels, and the confirmation of the heterothallic origin of the ascospores. Results seem to indicate that four progeny are *MAT-1*, two are *MAT-2* where as the remaining four show low levels of fertility that impede adequate *MAT* identification. Lack of funding and time did not allow us to confirm results obtained in crosses by *MAT*-PCR nor to sequence the *MAT* gene for phylogenetic analysis.

These ten strains are valuable seeing as only two strains (ATCC 15664 and BBA 62457) have been identified to date from international culture collections as belonging to this biological species (**BS 3**). The identification of these supplementary, more fertile, strains belonging to BS 3 should enable the determination of a sufficiently fertile *MAT-1/MAT-2* mating type tester couple that can constitute reference strains with the aim of establishing the presence of additional biological species within what was previously termed *G. xylarioides*.

Crosses between *G. xylarioides sensu lato* strains belonging to different BS produced protoperithecia (1), barren perithecia (2) or perithecia containing unidentified structures (3). Crosses with SG 4 representatives were either completely sterile (0) or produced protoperithecia (1) or barren perithecia (2). Crosses between the defined GxC BS/SGs and the GFC mating population (MP) mating type testers were completely sterile (data not shown).

3-3.2. Morphological diversity

Phenotype was used to analyze the possibility of rapid differentiation of biological species within the GxC by simple techniques. Macroscopic analysis of young PDA (Potato Dextrose Agar) and CA (Carrot Agar) cultures exposed to a light source allowed the observation of an orange pigmentation in *C. canephoralC. excelsa*-associated strains (BS 2, BS 3, BS 4) whereas *C. arabica*-associated strains (BS 1) did not produce this pigment (**Fig 2**). It should be noted that BS 3 and BS 4 strains are capable of producing '*F. udum*-like' colonies that produce wine red pigments. The similarity in the morphology of *G. xylarioides* and *F. udum* (*G. indica*) makes place for possible confusion between the two species when isolations are carried out from atypical hosts such as in Serani *et al.* (2007). Moreover, a growth temperature assay revealed that only strains belonging to SG 4 were capable of growth at 33°C on PDA (data not shown).

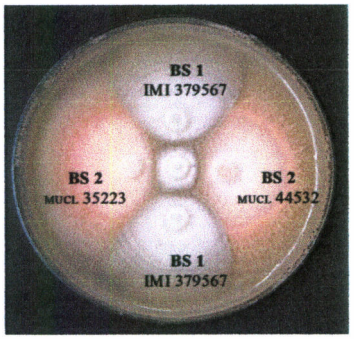


Fig 2 Lack of orange pigment production used to differentiate rapidly BS 1 from other representatives of the GxC. *MAT-1 C. canephora*-associated GxC (BS 2, orange colonies) mating type tester strains confronted with *MAT-2 C. arabica*-associated *G. xylarioides sensu lato* strain (BS 1, white colony) two weeks after inoculation on CA and a 7 day incubation under light banks.

Regarding the analysis of the teleomorph, sizes of perithecia and ascospores produced in GxC BS 1, BS 2 and BS 3 *in vitro* crosses are situated within the range described by Heim (1950) for perithecia observed on *C. excelsa* samples (**Table 2**). The extreme variability in ascospore shape and septation, as well as the importance of constriction at septa described by reference authors was equally observed. The majority of ascospores are one septate and can present a slightly larger upper cell, explaining the three sets of measurements given for each cross analyzed. When available, data is indicated under the form (min-) centil 0,05 - centil 0,95 (-max). *In vitro* produced ascospores appeared hyaline compared to "straw colored" mentioned for *in vivo* produced ones. As described by Saccas (1951), ascospores produced in GxC BS 3 revealed a pronounced constriction at the septa. Preliminary results indicate that the occurrence of ascospores with more than one septum and the intensity of the constriction at this level varies depending on the BS implicated in the cross. Furthermore, perithecia were produced on an abundant ramified stroma in crosses implicating BS 3 with a more discreet production for other BS. Moreover, preliminary data suggests that BS 1 perithecia are slightly larger in size than those produced in other crosses or in the literature. This calls for supplementary analysis of perithecia produced *in vitro* using other strains as well as perithecia isolated directly from *C. arabica* samples.

Table 2 Size of perithecia and ascospores obtained through *in vitro* crosses of members of the *G. xylarioides* complex and *G. indica* complex compared to reference descriptions by Heim & Saccas (1950) and Rai & Upadhyay (1982)

Species	Cross	Size (µm)		
		Perithecia	Ascospores	
			range	average
G. xylarioides complex				
Ref	***Coffea* spp. sample**	**200-400 x 180-300**	**12-20 x 4-5 x 5-5.6**	**NS**
BS 1	IMI 375909 x 389563	408 x 365	(10-)12-15(-16) x 4-5 x (4-)4.5-5.3(-6)	13.4 x 4.8 x 5.0
BS 2	MUCL 35223 x 43887	338 x 275	(11-)13-17(-20) x 4-5 x 4-5.6(-6)	15.3 x 4.8 x 4.9
BS 3	ATCC 15664 x BBA 62457	375 x 316	11-15 x 4-6 x 4.5-6	13.1 x 5.0 x 5.2

NS: not specified

3-3.3. Molecular diversity

Bearing in mind that crossings are a time-consuming assay that take at least 4 to 12 weeks to complete, and that the likelihood of identifying sexually compatible pairs can be reduced by a high proportion of wild field isolates that are either female sterile or completely sterile, it was crucial to find molecular techniques that could help identify promptly and with ease the "sexual compatibility group" or mating type (*MAT*) of any given isolate before confirmation in crosses. *MAT*-PCR, based on primers previously described in the literature for related ascomycetes, confirmed our hypothesis that ***G. xylarioides sensu lato*** is a **heterothallic** fungus. Moreover, this technique enables a rapid screening of the *MAT* ratio in the different regions surveyed and greatly reduces the number of crosses carried out. In the case of the coffee wilt pathogen, the observation of a balanced *MAT* ratio in all of the regions studied reflects the frequent observation of teleomorph in nature (with the exception of the Congolese Equator region where only *MAT-1* strains were sampled) suggesting that the sexual cycle plays a key role in the life cycle of the fungus.

Amplification of a small portion of the *MAT1-1* and *MAT1-2* idiomorphs was carried out initially with primers designed by Steenkamp *et al.* (2000) targeting respectively a restricted part of the *MAT1-1-1* (Gfmat 1a & 1b) and *MAT1-2-1* (Gfmat 2c & 2d) open reading frames (ORFs, **Fig 3**). Primers capable of amplifying respectively the entire *MAT1-1* idiomorph and the entire *MAT1-2-1* ORF of *G. xylarioides* strains were designed based on available sequences for related Fusaria. Available NCBI *F. oxysporum* (AB011379), *G. circinata* (AY21987), *G. subglutinans* (AY219878), and *F. guttiforme* (AY219875) sequences were aligned using ClustalW 1.82 (Pearson and Lipman, 1988) for the *MAT1-1* gene primer design. Primers Gxp5', Gxp8, Gxp9, Gxp11 and Gxp13 as well as internal primers used for sequencing were designed from highly conserved regions of the aligned sequences. Similarly, *F. oxysporum* (AB011378), *G. fujikuroi* (AF100926) and *G. circinata* (AF194869) sequences were aligned for *MAT1-2* Gxp21 primer design.

Amplification of the entire ***MAT1-1* idiomorph** in *G. xylarioides* strains can now be carried out via three distinct PCRs using primer couples Fo14 (Arie *et al.*, 2000)/Gxp5', Gxp11/Gxp9, and Gxp8/Gxp13 (**Fig 3A**). A 4749 to 4803 base pair (bp) fragment was sequenced for *G. xylarioides sensu lato MAT-1* strains MUCL 35223, MUCL 44532, Gx3P22, IMI 375907, BBA 62458, ATCC 15664, MNHN 709 as well as for closely related *Fusarium* sp. strains IMI 271070, NRRL 22540 and NRRL 26064.

Amplification of the entire ***MAT1-2-1* ORF** and a portion of the 3' flanking region was carried out with primer pair Fo14 (Arie *et al.*, 2000) or Gfmat2d Steenkamp *et al.* (2000)/Gxp21 (**Fig 3B**). A 1198 to 1382 bp fragment was sequenced for *G. xylarioides sensu lato MAT-2* strains OUG 008, MUCL 43887, MUCL 44549, IMI 204746, IMI 375908, IMI 379567, CBS 25852, CBS 74979, BBA 62455, BBA 62457 as well as for closely related *Fusarium* sp. strains IMI 193652 and IMI 275452.

MAT sequence analysis against EMBL/NCBI databases revealed that *G. xylarioides sensu lato* is nested within an important and pathogenic *Fusarium* complex, the ***Gibberella fujikuroi***

Complex (**GFC**). Moreover, the distinct PCR primer sets designed for the amplification of the *G. xylarioides sensu lato MAT* idiomorphs were successfully implemented to nine of the ten known GFC mating population (MP A to H) tester pairs in addition to *F. oxysporum* and *G. indica* strains (**Fig 3**).

Phylogenetic analysis of ***MAT*** sequences resolved four distinct clades (phylogenetic species, PS) corresponding to the four BS/SGs defined in crossing assays (**Fig 4A & 4B**). Amplification and sequencing of **unlinked autosomal** (non-*MAT*) **nuclear genes** such as translation elongation factor 1-α (*tef*), calmodulin (CL) and histone 3 (H3) were equally carried out for use in phylogenetic analysis (**Fig 4C**). Results indicate that the individual gene trees inferred are non concordant with each other as well as with the *MAT* trees generated. Only the combined autosomal gene tree reflects the distinct clades resolved in the *MAT* phylogenetic analysis. The placement of the GxC as a sister clade to the *F. udum*/*Fusarium* spp. complex is consistent. Results suggest a differential power of resolution depending on the gene used and question the pertinence of all genes for phylogenetic inference and speciation.

Moreover, analysis of the **left idiomorph flanking region** (**Fig 5**), theoretically common to both mating types, revealed striking observations. The most unusual is the presence of a 60 bp region (marked by a red line) containing **mating type-specific sequences** common to both the *G. xylarioides* and the *G. indica* complexes. This region harbours very few species- and/or mating type-specific mutations. Surprisingly, *C. arabica*-associated *G. xylarioides sensu lato MAT-1* strains **IMI 375907** and **BBA 62458** possess the sequence type for *MAT-2* strains in this region.

Concurrently, a global picture of the GxC population structure was obtained by **RAPD** highlighting the presence of four distinct haplotypes corresponding to BS/SGs defined (data not shown).

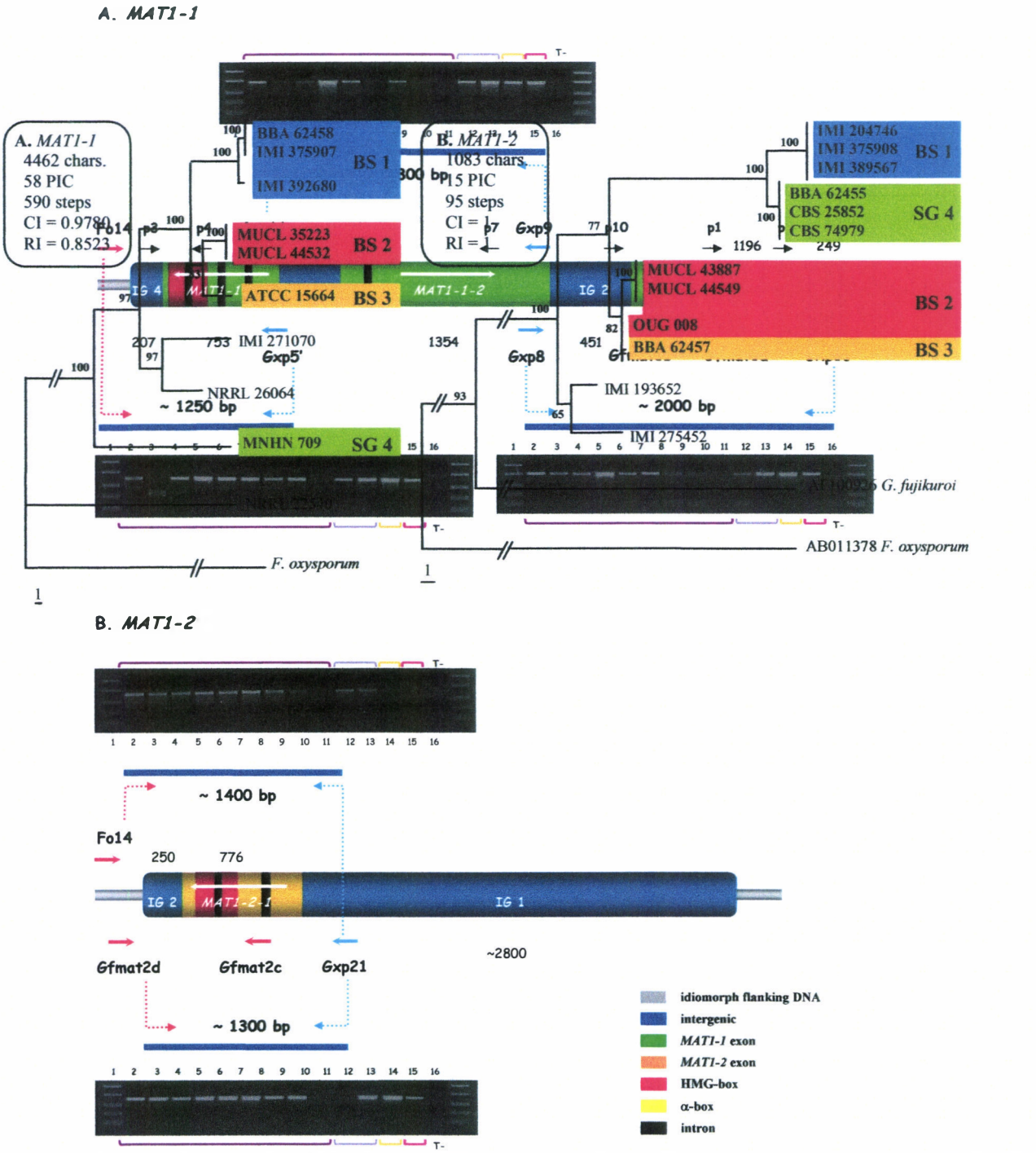
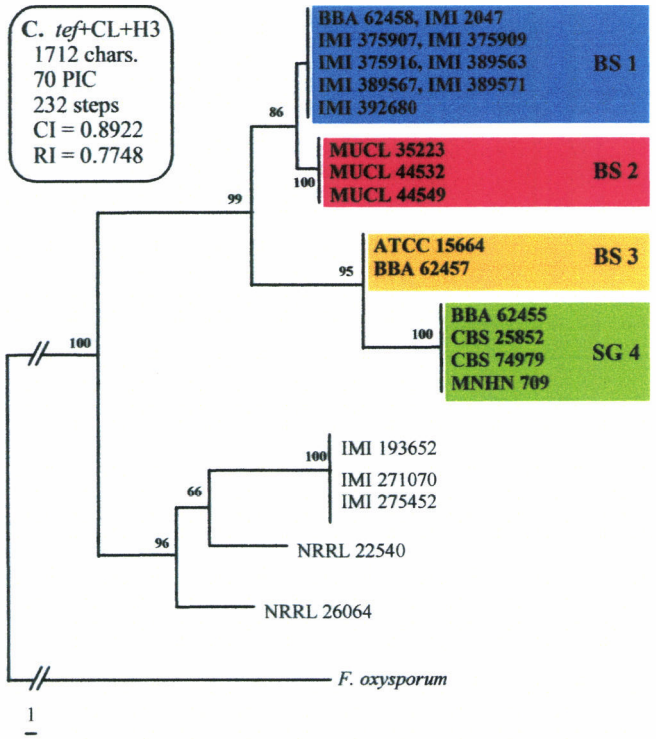


Fig 3 Map of the mating type locus (*MAT*) of *G. xylarioides* and *G. indica sensu lato*. (**A**) *MAT1-1* (~4605bp) and (**B**) *MAT1-2* (~3824bp) idiomorphs drawn to proportion showing introns, exons, conserved domains, intergenic regions (arbitrarily designated IG 1, IG 2, IG 3 and IG 4) and flanking DNA. A white arrow indicates the direction of transcription of each of the four *MAT* ORFs. The centromere is situated on the left of the figure. PCR strategies for amplification of the *G. xylarioides sensu lato MAT* loci are described. Red arrows represent previously described primer sets, blue and short black arrows are respectively PCR and sequencing primers designed in this study. PCR gels obtained with designed primers using GFC and *F. oxysporum* strains are shown. Lanes 1 and 16 correspond to Fermentas Middle Range ladder indicating 100 bp, 400 bp, 850 bp, 2000 bp and 5000 bp. Lanes 2 to 10 correspond to GFC MPs A to I, lanes 11 and 12 are *F. oxysporum*, lane 13 represents *G. indica sensu lato*, lane 14 corresponds to *G. xylarioides sensu lato*, and lane 15 is a negative control.

Fig 4 Maximum-parsimony phylograms based on (**A**) entire *MAT1-1* idiomorph sequence, (**B**) partial *MAT1-2* idiomorph sequence and (**C**) combined autosomal (*tef*+CL+H3) datasets for representatives of the *G. xylarioides* (GxC) and *G. indica* (GiC) complexes from diverse geographical and host origins. Putative phylogenetic species (PS) corresponding to biological species (BS) and sterility groups (SG) identified in crossing assays are indicated by a colored box next to the terminal clades resolved. Trees were generated with PAUP* v.4.0 b10 (Swofford, 2001) using *F. oxysporum* as outgroup. Bootstrap values are indicated in percentages at internodes when replication frequencies exceed 60%.



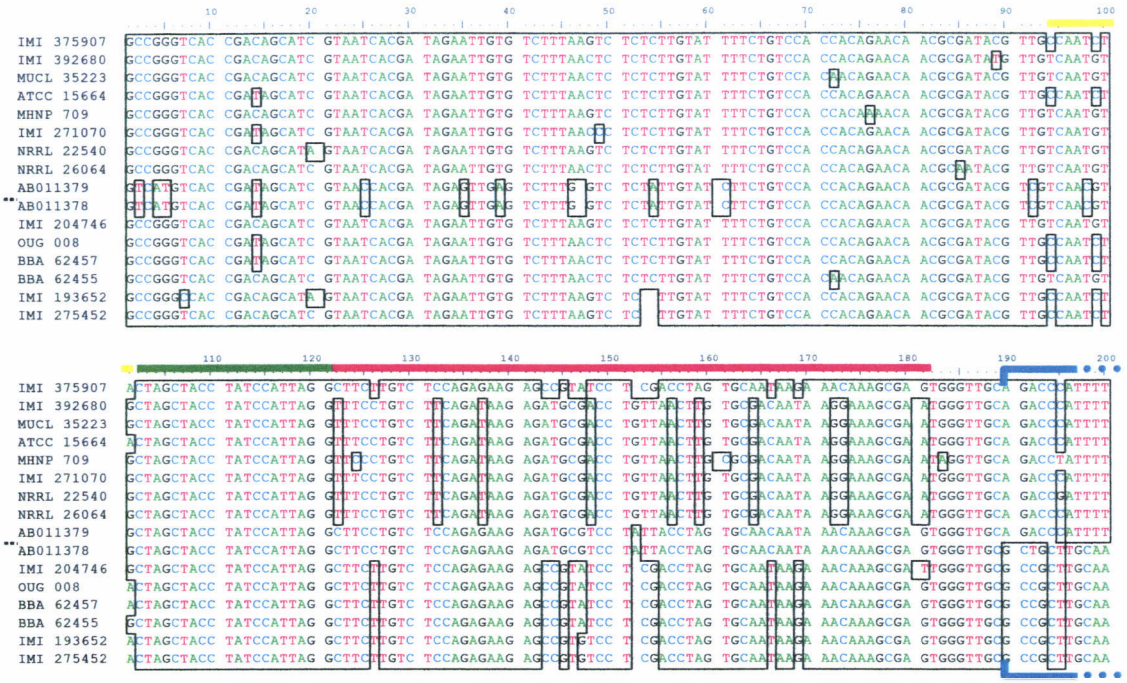


Fig 5 Sequence alignment of representative *MAT1-1* and *MAT1-2* flanking regions situated to the left of the idiomorph for members of the *G. xylarioides* and *G. indica* complexes along with *F. oxysporum* (AB011378 and AB011379). *MAT-1* and *MAT-2* strains are separated by a dotted line and sequence similarities in sequenes are boxed. The beginning of the idiomorph is indicated by a blue line, the putative 60 bp *MAT*-specific region bordering it by a red line, a conserved 20 bp region is lined in green and an ambiguous 8 bp stretch in yellow.

4. Problems encountered

Pathogenicity tests were not pursued due to the "heaviness" of coffee cultivation and the fact that interpretation of results was ambiguous.

5. Technology implementation plan

6. Publications and papers

- Tshilenge-Djim, P., F.T.J. Munaut, A. Kalonji, and H.M.M. Maraite. 2004. Caractérisation des *Fusariums* spp. associées au dépérissement du caféier Robusta en République Démocratique du Congo. Parasitica **60**: 67-82.
- Lepoint, P.C.E., F.T.J. Munaut, and H.M.M. Maraite. 2005. *Gibberella xylarioides* from *Coffea canephora*: a new mating population in the *Gibberella fujikuroi* species complex. Appl. Environ. Microbiol. **71 (12):** 8466-8471.
- Lepoint, P.C.E. December 2006. Speciation within the African coffee wilt pathogen. Thesis. Université catholique de Louvain. 208pp.
- Lepoint, P.C.E. Characterization of the *MAT1-1* and *MAT1-2* idiomorphs in the *Gibberella xylarioides* and *Gibberella indica* Species Complexes (*In preparation for Fungal Genetics and Biology*).
- Lepoint, P.C.E., C.A. Decock, F.T.J. Munaut, and H.M.M. Maraite. Speciation within the *Gibberella xylarioides* and *Gibberella indica* Species Complexes (*In preparation for Fungal Genetics and Biology*).
- Lepoint, P.C.E., C.A. Decock, F.T.J. Munaut, and H.M.M. Maraite. Neotypification of *Gibberella xylarioides* (*Fusarium xylarioides*) (*In preparation for Taxon*).

7. Conclusions

Results suggest that *G. xylarioides sensu lato* is a **heterothallic fungus** composed **primarily** of **hermaphroditic** strains, and that Booth's (1971) observation of sex-linked morphological

characteristics is erroneous. This explains the ease with which the perfect form is observed in nature provided that environmental conditions are favorable and could explain why we have not been able to observe the teleomorph in our monosporic artificial inoculations.

It would appear that what was previously circumscribed as being **"*G. xylarioides*"** entails in reality **four distinct biological species/sterility groups** that are resolved as being distinct PS using a combined gene analysis method. It is thus more pertinent to speak of the ***G. xylarioides* complex (GxC)** or *G. xylarioides sensu lato* when speaking of an unidentified isolate. Two of these species (BS 3 and SG 4) have not been isolated since the first coffee wilt epidemic in the 1950s where as BS 1 (limited to *C.arabica* in Ethiopia) and BS 2 (found on *C. canephora* in the Congo basin region) are responsible for the current epidemics (**Fig 6**). These novel results highlight the importance of adapted quarantine measures necessary for limiting the sympatric occurrence of these species and their possible recombination. The exact status of the different BS/SGs as subspecies or species is still under discussion.

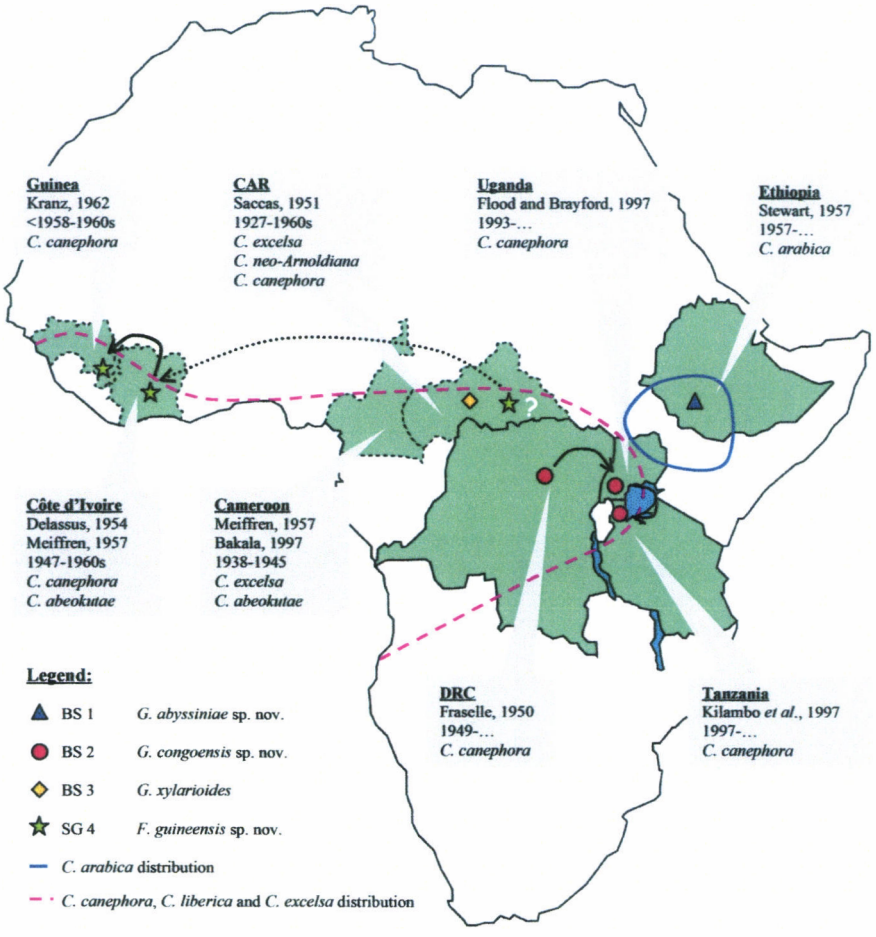


Fig 6 Distribution of Coffee Wilt Disease (CWD) across Africa since its first report on *C. excelsa* in 1927 in the Central African Republic (CAR, former Oubangui-Chari) to present. There has been no report of CW in the past 50 years from countries delimited by a dotted outline; those currently affected are delimited by a full outline. For each country, data concerning dates of initial observation of CW, *Coffea* species affected, and bibliographical references are indicated. Biological species (BS) and sterility groups (SG) defined in this study with proposed UCL nomenclature, putative paths of dissemination (indicated by a black arrow) and *Coffea* spp. distribution (Charrier and Eskes, 2004) are equally indicated.

INDIVIDUAL FINAL REPORT

PARTNER P3: National Agricultural Research Organization (NARO)
UGANDA

Dr Pascal Chungason Musoli
Dr Georgina Hakiza

Introduction

Coffee wilt disease (CWD) caused by *Fusarium xylarioides* was a serious menace to Robusta coffee (*Coffea Canephora)* and *Coffea liberica* spp. in Africa during 1920s to 1960s (Guillemat, 1946; Fraselle, 1950; Saccas, 1951; Muller, 1997). But re-planting using resistant varieties seemed to have eradicated it on *C. canephora* and *C. liberica* spp. in the Central and West African countries during 1960s and 1970s. However in the early 1980s, CWD was reported again on *C. canephora* in the Democratic Republic of Congo (DRC), from where it spread to Uganda and Tanzania, affecting the same coffee species. Coffee wilt disease has remained endemic to *Coffea arabica* in Ethiopia since it was first reported in 1957 (Lejeune, 1958).

This disease was reported for the first in Uganda in 1993 (Flood, 1997) on *C. canephora* in Bundibugyo, a district that borders the Democratic Republic of Congo. There after the disease rapidly spread to all parts of the country, affecting only *C. canephora* even in areas were *C. Arabica* is cultivated side by side with *C. canephora*. By 2002 CWD was present on 90% of the *C. canephora* farms in Uganda and it had destroyed 44.5% of the *C. canephora* trees countrywide (Oduor, 2005), leading to farmers' desperation and dire need for its control. Given that the disease was appearing in Uganda for the first time, there were no resistant varieties for planting/re-planting in affected areas. Phytosanitory controls emphasized at the beginning of the control campaign were not effective in preventing the disease spread and were difficult to implement by the farmers. Chemical control was considered ineffective since it had failed in previous attempts in Central and West African countries (Muller, 1997). It was therefore considered necessary to develop CWD resistant *C. canephora* commercial varieties for the planting/re-planting programmes. However prerequisite information required for designing an effective breeding strategy to develop varieties with durable resistance was lacking. In this regard, studies were carried out under the different schematic Work Packages of the INCO-COWIDI project '**Development of a long-term strategy based on genetic resistance and agro-ecological approaches against Coffee Wilt Disease**' to generate such prerequisite information. The research was carried out collaboratively between CIRAD (France) and the Coffee Research Institute (CORI) of the National Agricultural Research Organisation (NARO) in Uganda. Major research activities carried out in this project and their main findings are given in this report. Follow up activities for implementation of the obtained results in developing lasting solution for CWD in Uganda are also described.

1- Objectives

1- To understand the variability of the CWD pathogen in relation to its life cycle, host variety and geographical location
2. To develop appropriate and cost effective methods of screening coffee germplasm for resistance against CWD
3. To identify sources of resistance for controlling CWD in *C. canephora* in Uganda
4- To understand host/pathogen interaction and isolate aggressiveness using artificial inoculation tests (different inoculation methods).
5. To understand the inheritance of CWD resistance in *C. canephora*
6. To understand the genetic diversity of available *C. canephora* genetic resources that could be exploited for resistance to develop CWD resistant commercial varieties.
7 To understand the spatial and temporal spread of CWD and to understand the longevity and survival of CWD pathogen

2- Activities

2-1- Variability of CWD pathogen in relation to its life cycle, host variety and geographical location

2-1-1 Collection and dispatch of the CWD pathogen specimens for studies in European laboratories

Samples of stem parts of *C. canephora* trees with CWD symptoms were collected from various parts of the *C. canephora* trees and possible alternate hosts, in different affected regions. Parts of the samples were maintained in the laboratory at CORI and some parts were dispatched to European laboratories (CIRAD, CABI and Louven University) for studying the genetic variability and sexual cycles of the pathogen population.

2-1-2 Description of the CWD pathogen life cycle

Production of perithecia on naturally infected coffee trees in the field was monitored on *C. canephora* clones 1s/2, 1s/3, 1s/6, 223/23 and 258/24 grown in Block 13 at CORI-Kituza. The data was collected at 4 weeks intervals starting from March 2003. The trees for this study were selected according to the disease levels as follows:
Score 1 – First symptoms (leaf wilting/dropping on one stem tip, less than 1/3 from top)
Score 2 – Symptoms on more than half of stem height
Score 3 – The whole stem(s) wilted
A grid was placed on 3 marked positions along the selected tree stem. The grid positions were:

Top 65 cm from ground level
Middle 35 cm from ground level
Bottom 5 cm from ground level.

2-2- Development of appropriate and cost effective protocols for screening coffee germplasm for resistance against CWD

2-2-1Development of a standard inoculation method

In order to be able to screen a large numbers of Robusta coffee plant materials for resistance to coffee wilt disease, it was essential to develop a standardized method of artificial inoculation to detect resistance. Three methods of inoculation using 3 different inoculum concentrations (10^6, 10^5 and 10^3 spores/ml) on half sib progenies of 3 different *C. canephora* clones/varieties (J/1/1-resistant to CWD; 1s/3 - moderate resistance; P/3/6 - susceptible to CWD under field conditions) at 9 months old were compared.

Inoculation by root dipping

Plants were first watered well, and then gently removed from their bags/pots. Soil attached to the root system was gently removed under running water prior to placing in the inoculum suspension for 2 hours. The treated plants were replanted in sterile soil, watered and placed under polythene cover for 24 hours after which they were placed randomly in the green house.

Inoculation by stem injection

The Inoculum was introduced into green part (where bark has no yet started forming i.e the second internodes following the first pair of leaves) of the stem of each plant using a small needle (less than 1 mm diameter) attached to a hypodermic syringe (5 ml). A drop of about 30 ul was injected in each stem. The inoculated plants were placed under a polythene sheet (to maintain high humidity) for 24 hours, after which the plants were randomly placed in the screen house.

Inoculation by stem wounding

A shallow cut was made on the stem with a razor blade or scalpel just below the second internode and a drop of the standard inoculum placed in the wound. The inoculated plants were carefully kept under

polythene cover as for syringe injection above and later arranged randomly as before. Control plants were inoculated with sterile distilled water. All plants were maintained in the screen house and closely monitored for wilt symptoms for 120 days. The experiment was repeated once.

2-2-2. Defining other protocols suitable for assessing coffee trees for resistance to CWD

Assorted protocols (resistance traits/indicators, plant materials (seedlings, cuttings), study environments (field, screen house, climatic chamber) and inoculation techniques were evaluated in regard to CWD resistance in *C. canephora* trees so as to develop an effective and reliable protocol for quantifying resistance among coffee trees. Analysis of disease data collected on *C. canephora* trees in the field and artificial inoculations (screen house & climatic chambers) was used to compare the resistance in the field and in artificial inoculations. The same data was used to compare the resistance measured using different indicators (mortality, symptom severity index, disease period and area under disease progress curves).

2-3- Identifying sources of resistance to CWD

Twenty *C. canephora* clones were studied in a field trial and in artificial inoculations (as rooted cuttings) plus their half sib progenies studied in the screen house and climatic chambers. Half sib progenies of different *C. canephora* populations (wild, feral, cultivated, isolated) were also studied for resistance to CWD in artificial inoculations. Disease data collected from the inoculated plants was used to analyze the resistance of the different populations to CWD.

2-4- Host-pathogen interactions

These studies were carried out to understand the interaction of Ugandan *C. canephora* with *F. xylarioides* strains affecting *C. arabica* in Ethiopia and historical strains collected from *C. liberica spp*. The plants were inoculated with *F. xylarioides* isolates CAB003 (*C. canephora* strain), CAB007 (*C. arabica* strain) and DSMZ (*C. liberica* type excelsa strain). The analysis of disease data collected from the artificially inoculated plants studied in controlled climatic chambers provided the information on the response of *C. canephora* to *F. xylarioides* isolates causing coffee wilt disease in *C. arabica* and *C. liberica spp.*

2-5- Inheritance of the resistance to coffee wilt disease in *C. canephora*

Breeding for CWD resistance is now the main objective of *C. canephora* improvement program in Uganda but the genetic systems controlling CWD resistance in this species was not known prior to this study. Analysis of disease data collected from *C. canephora* clones and half diallel progenies in field trials at Kituza generated the information on resistance and inheritance of the resistance to coffee wilt disease in *C. canephora*.

2-6- Genetic diversity of Ugandan *Coffea canephora*

In this study the genetic diversity of Ugandan *C. canephora* genotypes of wild and cultivated origins was analyzed in comparison to genotypes from the previously described diversity groups (Dussert et al. 2003). In this investigation, we addressed four questions: 1) what is the genetic diversity and structure of wild and cultivated Ugandan *C. canephora*? 2) Where are Ugandan genotypes located in the global *C. canephora* diversity? 3) What are the implications of the findings to the conservation and utilization of the Ugandan *C. canephora* genetic resources in breeding for resistance against coffee wilt disease? In this regard, *C. canephora* populations from two primary forests (Kibale and Itwara) and Kalangala islands in Lake Victoria in Uganda were explored for variability in search for potential sources of resistance to control CWD. The variability of these populations was studied in comparison to that of all the other previously described groups using molecular markers. DNA data from 196 Ugandan *C. canephora*

genotypes of wild and cultivated origins was analyzed in comparison to that of genotypes from the previously known *C. canephora* diversity groups using 24 microsetallite markers.

2-7- Spatial and temporal spread of CWD

2-7-1 On-farm activities

Observations started in 2002 on development and spread of CWD at selected smallholder Robusta coffee farms in Mayuge and Iganga districts in Eastern Uganda and Masaka and Rakai districts in Central Uganda. Two farms were selected per district. Data were collected from each site at intervals of 4 or 8 weeks. The data (wilt incidence and severity) were recorded in specific areas and coffee trees (8 rows x 16 rows = 128 trees) within the farms.

2-7-2 On-station activities at Kituza

Studies were carried out on a *C. canephora* field experiment planted in October 1997at CORI, Kituza.in Uganda. The trial had 20 clones. 16 out of the 20 clones were single tree selections among progenies of specific crosses and the remaining four (1s/2, 1s/3, 223/32 and 257/53) were commercial clones. The degree of CWD infection was measured by assessing percentage plant defoliation caused by the disease, twice every month on a disease symptom severity scale of 1 to 5 where 1 = no disease, 2 = 1-25% defoliation, 3 = 26-50% defoliation, 4 = 51-75% defoliation, 5 = 76-100% defoliation. Plants in level 5 were considered dead. The Coffee wilt disease was first observed in the experimental field in 1999 but systematic assessment start in April 2001 and lasted until March 2006. From March 1999 to Match 2001, all affected trees were uprooted to minimize and, if possible, eradicate the disease from the trial field.

Percent tree mortality was calculated from the disease severity data as a proportion of plants that attained level 5 on the disease severity scale. Maps showing the spatial distribution of diseased (sick and or dead) coffee trees at annual intervals were generated from the disease symptom severity data using SAS (SAS Institute Inc., Cary, NC, 1989). The maps were used to visualize the spread of this disease over time. The Geo-statistical analyses (semi-variance and kriging) were performed on the data to show the spatial-temporal structure of disease

2-8- Longevity/survival of the CWD pathogen

Different experiments were developed to evaluate the duration of the survival in the soil and in infected stem pieces, and the role of farm tools in transmission of CWD.

3- RESULTS

3-1. Variability of CWD pathogen in relation to its life cycle, host variety and geographical location

3-1-1 Collection and dispatch of the CWD pathogen specimens

Single spores isolates or infected pieces of wood collected from the various parts of Ugandan (Figure 1) were submitted to CABI UK or/and CIRAD for molecular studies. In addition, isolates from specific clones were sent to CIRAD.

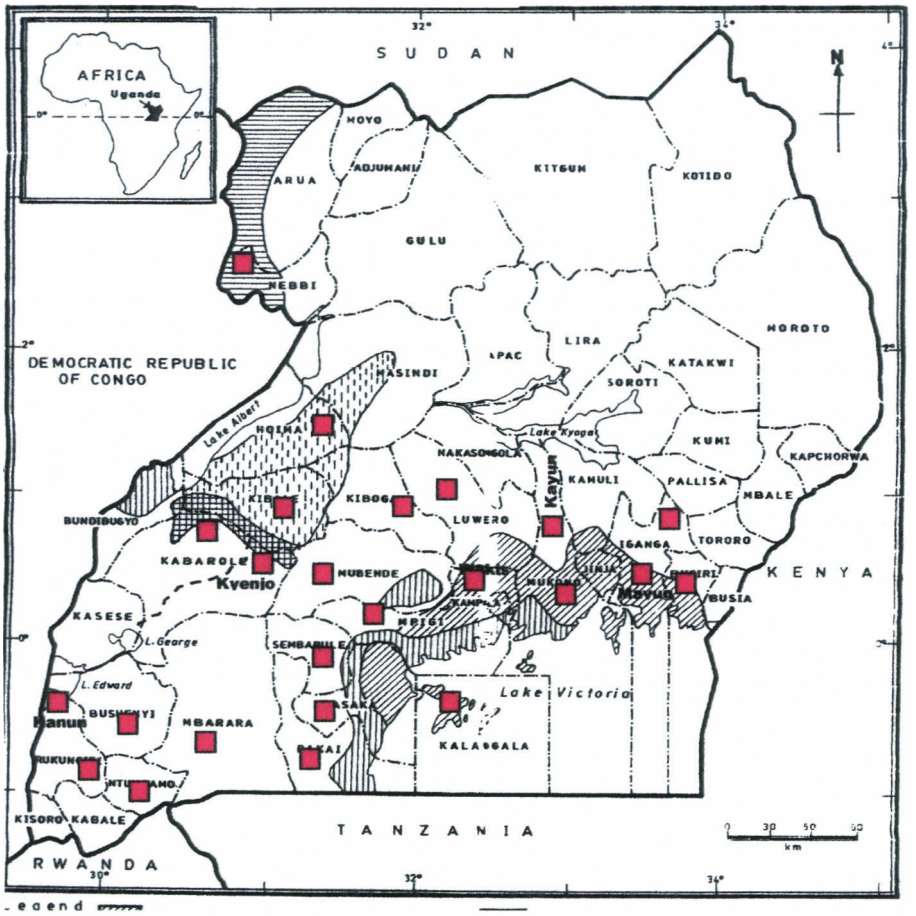


Figure 1: Map of Uganda showing locations (■) from where *F. xylarioides* isolates

3-1-2- Description of the CWD pathogen life cycle

For all the clones, perithecia were rarely produced at the top grid but they were abundant at the bottom grid. The perithecia were not observed during dry periods but they started forming within 2 weeks of the rainy days and after the death of infected trees. Perithecia formation began from the bases of the stems and progressed upwards. Off-season rains (short spells of rain during a typical dry season) can also induce formation of perithecia in the field. Such spells are common at Kituza and in a coffee field there is always a lot of inoculum. Discarded dry inoculated seedlings or rooted plants do not form perithecia unless they are watered. Perithecia on such plant materials were confined to the collar regions and did not extend along the stems.

The pathogen is found in soil although direct isolation from soil was not possible. It is assumed the pathogen (in form of ascospores, mycelial pieces or conidia?) enter the host tissue through the roots and establishes itself in the xylem vessels causing blockage and hence wilting of the plant only on the affected side. When the affected stem dries up, numerous perithecia are formed mostly on the lower part of the stem above the earth surface but a few can form below the ground, where the blue_black staining is present. Infection can also occur through wounds on the lower part of the stems. These wounds are normally inflicted during weeding using hoes or machetes. In the screen house, wounds inflicted higher up the plant do not result in full-blown wilt symptoms. In such cases, the affected plant develops symptoms above the point of entry and eventually dries up but the lower portion of the stem remains unaffected.

Inoculum from diseased coffee plants is spread by people as they transport the infected plants for firewood, grazing animals, and wind or washed into soil by rain to form soil inoculum or moved by run-off. The disease manifestation is faster during the rainy season because the plants are very active. During dry season due to moisture stress, disease progress within the plant is slow.

As a control measure it is recommended that farmers uproot infected plants at the earliest symptoms in order to eliminate sources of infection. However, many farmers do not uproot but cut plants down leaving stumps. Perithecia therefore continue to be formed on the remaining stumps and infection is spread to plants within the field or at distances away.

3-2- Development of appropriate and cost effective protocols for screening coffee germplasm for resistance against CWD

3-2-1- Development of a standard inoculation method

The screen house inoculations are a good indication of adult plant reaction to the disease. This is further supported by the fact that, to date very few plants screened using this method have developed CWD symptoms under field conditions. Root dip was selected because of its consistent trends in latent periods and wilt incidences in the several experimental tests as compared to stem inoculations (stem injection and stem wounding) and soil drenching.

Table 1: Comparison of the disease incidence on 4 *C. canephora* clones infected with CWD under the screen house (artificial) and in one of the fields (natural infection) at Kituza

Clone	Screen house inoculation (Mean % incidence after 90 days).	Field infection (Mean % incidence during Jan. 2002 – Nov. 2006
1s/2	88.7	93.3
1s/3	100	95.1
1s/6	94.3	92.7
258/24	94.3	98.4

3-2-1- Defining other protocols suitable for assessing coffee trees for resistance to CWD

The results from the analysis on field data revealed that percent plant mortality was the best trait for measuring resistance to coffee wilt disease, although area under disease progress curve and disease symptom severity rated on a standardized scale such as the 1-5 scale (1 = no disease and 5 = dead tree) can also satisfactorily quantify resistance. Disease period (time from observation of initial disease symptom to when the plant is declared dead) is not a good trait for measuring resistance, since some susceptible genotypes have longer disease periods than resistant genotypes and some relatively resistant genotypes have shorter disease periods than some susceptible genotypes. The studies found a strong relationship between genotype resistance given in artificial inoculations (screen house and climatic chamber) and in the field, especially where the artificially inoculated plants are cuttings. This implies that artificial inoculation are ideal for assessing trees for resistance to CWD since the technique can handle many young plants in a small area and results are obtained within 3-4 months as compared the 5-7 years of field study. There is significant correlation between half sib progenies studied in artificial inoculations and their parents studied in field trials

3-3- Sources of resistance to CWD

Evaluation of different Ugandan *C. canephora* clones, progenies and populations for CWD resistance using percentage of mortality data revealed that genotypes and progenies respond quantitatively to CWD infections. This shows that CWD resistance among *C. canephora* is controlled by many genes. Through these studies two wilt resistant clones (Q/3/4 and J/1/1) were identified, which can be released for commercial cultivation if they qualify after multi-location evaluations (Figure 2). It was also revealed from these studies that commercial clones 257/53, 1s/2 and 1s/6 are very susceptible to CWD and therefore they should be removed from the commercial list.

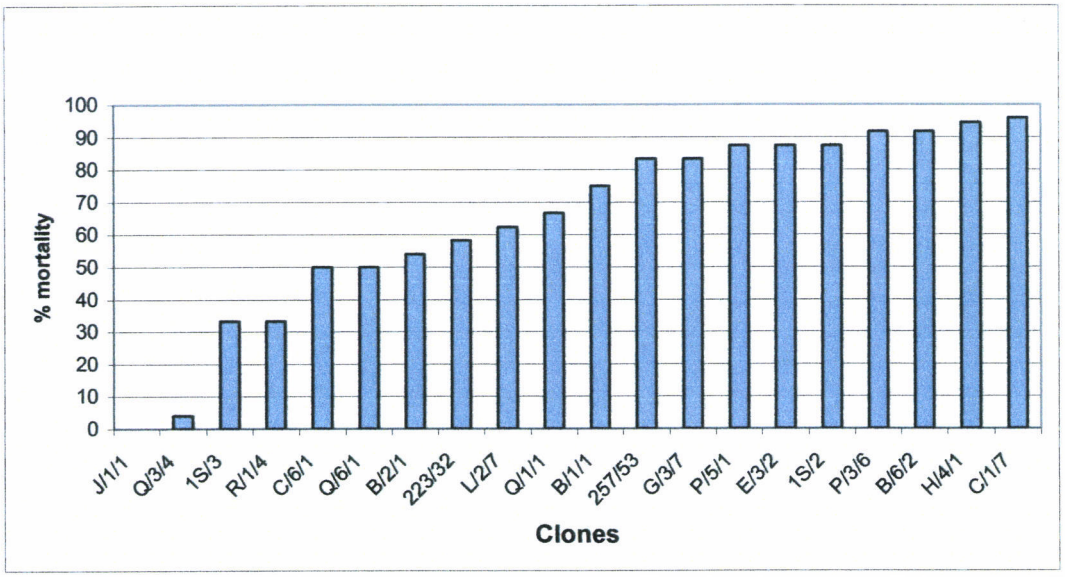


Figure 2: Percent mortality among *C. canephora* clones after 7 years in CWD infected field

Analysis of disease data on *C. canephora* half sib progenies from different populations (wild plants from Itwara and Kibale forest, feral trees from Kalangala islands isolated in Lake Victoria and cultivated trees of Erecta and Nganda sub-groups) studied in artificial inoculations was used to assess the level of CWD resistance among wild, feral and cultivated populations. This information is valuable for sourcing resistance among diversity sub-groups within Ugandan *C. canephora* population. It was revealed from this study that Kalangala and Itwara populations were highly resistant to CWD, highlighting the importance of these populations as sources of resistance genes for inclusion in the commercial varieties (Figure 3). The Nganda and Erecta populations were very susceptible, while Kibale progenies were moderately susceptible. Significant variability was also observed within each of these populations.

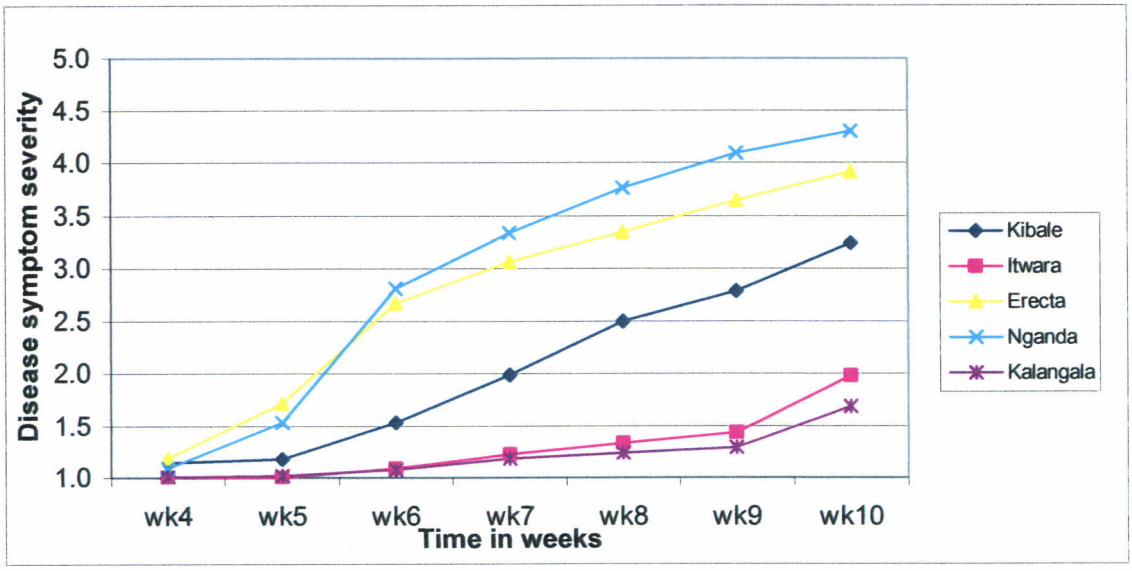


Figure 3: Variation of susceptibility to coffee wilt disease among half sib progenies of different *C. canephora* populations.

Beside these specific populations/genotypes (the clones in field trials and specific progenies highlighted above which were studied in screen house and climatic chamber), rooted cuttings and half sib progenies of many *C. canephora* genotypes in the germplasm collections at CORI and trees surviving among farms devastated by the CWD were artificially tested in the screen house for resistance. Through this process, over 1500 resistant individual trees were identified and they have been planted in mother gardens for multiplication and field evaluations (for resistance to diseases, quality and yield) as clones.

Artificial tests performed on seedlings progenies of various *C. arabica* varieties found this species resistant to CWD in Uganda. Similar tests performed on F1 Arabusta (*C. arabica* x *C. canephora*) clones found the Arabusta genotypes resistant to the CWD in Uganda. This confirmed that CWD is not a problem of *C. arabica* and this species together with the F1 Arabusts can be used as sources of resistance genes to control CWD of *C. canephora* in Uganda

3-4- Host-pathogen interactions

It was revealed from these studies that *F. xylarioides* isolate CAB003 (isolate from *C. canephora*) causes severe mortality to *C. canephora* followed by *C. liberica* isolate DSMZ62457 (Figure 3). *C. arabica* isolate CAB007 is not lethal to *C. canephora (Figure 4)*. *C. liberica* isolate DSMZ62457 causes severe mortality to *C. liberica* type excelsa but isolates *C. canephora* isolate CAB003 was not lethal to this species (Figure 5). These results imply that the pathogen populations that are affecting C. arabica in Ethiopia and *C. canephora* in Uganda, Tanzania and DRC are different from each other and they are also different from the pathogen population that caused the historical disease on *C. liberica* spp. The results also imply that *C. liberica* type excelsa can be a source of resistance to control CWD in *C. canephora* and *C. canephora* can be a source of resistance to control CWD in *C. arabica*. This information is particularly important in selecting resistant varieties for use in different agro-ecological areas infested with different *F. xylarioides* isolates and it is a prerequisite for interspecific hybridization when introgressing CWD resistance from one species into the other. It is also important for designing effective germplasm exchange and quarantine strategies.

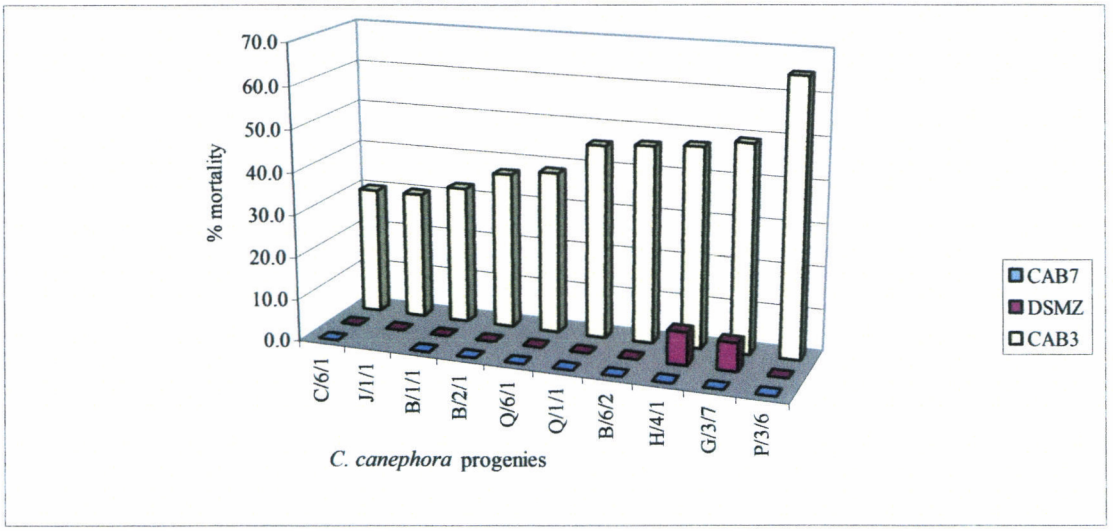


Figure 4: Mortality among *C. canephora* half sib progenies inoculated with *F. xylarioides* isolates CAB003, CAB007 and DSMZ62457

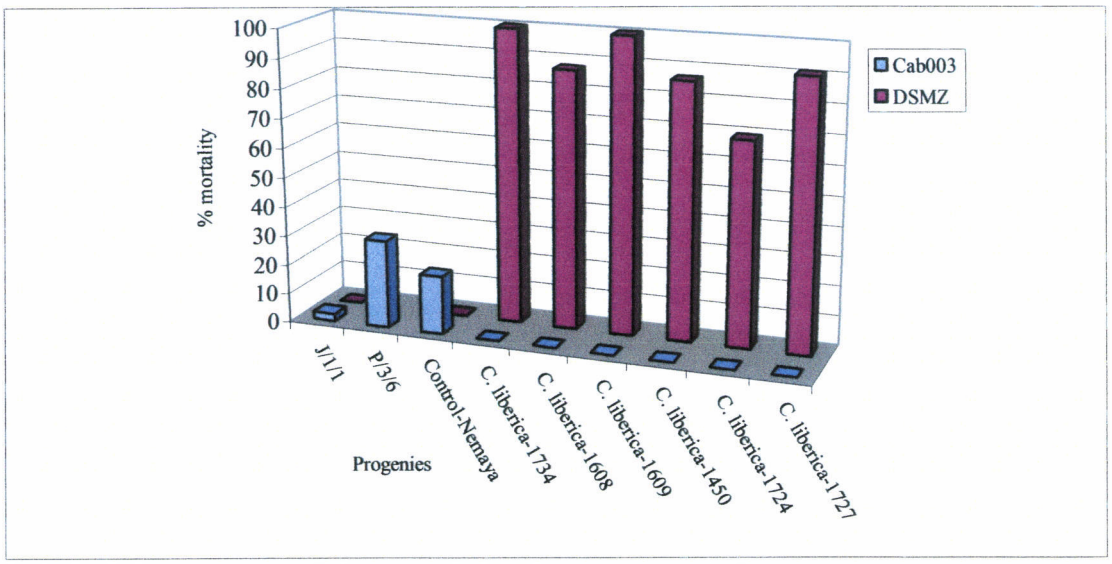


Fig.5 Mortality among *C. canephora* and *C.* liberica var. *dewevrei* half sib progenies inoculated with *F. xylarioides* strains CAB003 and DSM62457J/1/1 and P/3/6 are *C. canephora* progenies from Uganda.Nemaya is *C. canephora* progeny provided by CIRAD.

C5- Inheritance of the resistance to coffee wilt disease in *C. canephora*

The inheritance was calculated from the different experiment at a plant mortality of 50-65%, given that the resistance was quantitative. Broad sense heritability calculated from the disease data of the half diallel progenies was moderate (0.329 and from the clones in the field was also moderate (0.333). Narrow sense heritability calculated from the half diallel progenies was low (0.112) and narrow sense heritability estimated by regression of half sib progeny means onto parent means in the field and screen house were moderate (0.183 and 0.369 respectively). This shows that CWD resistance is heritable and therefore progenies of crosses between susceptible and resistant progenitors can have better resistance than their susceptible parents. Thus resistance of Uganda current commercial clones, which are susceptible to CWD, can be improved by hybridising with the resistant genotypes. However because *C. canephora* is predominantly out breeding and genotypes are heterozygous, progenies of theses crosses are expected to be heterogeneous, with variable levels of CWD resistance and other required agronomic traits. Individual progeny trees can be evaluated and before selecting superior genotypes for propagation as commercial clones.

C6- Genetic diversity of Ugandan *Coffea canephora*

Analysis of DNA from different Ugandan *C. canephora* populations revealed that *C. canephora* in Ugandan is genetically very diverse and it is variable from the previously known diversity groups, thus it forms another diversity group within the species (Figure 6). High genetic diversity within this species highlights the high potential of deriving genes for developing improved commercial varieties, including resistance to coffee wilt disease. For effective conservation of the diversity of *C. canephora* genetic resources, sample genotypes from the different diversity population could be conserved in different national and international conservation centres.

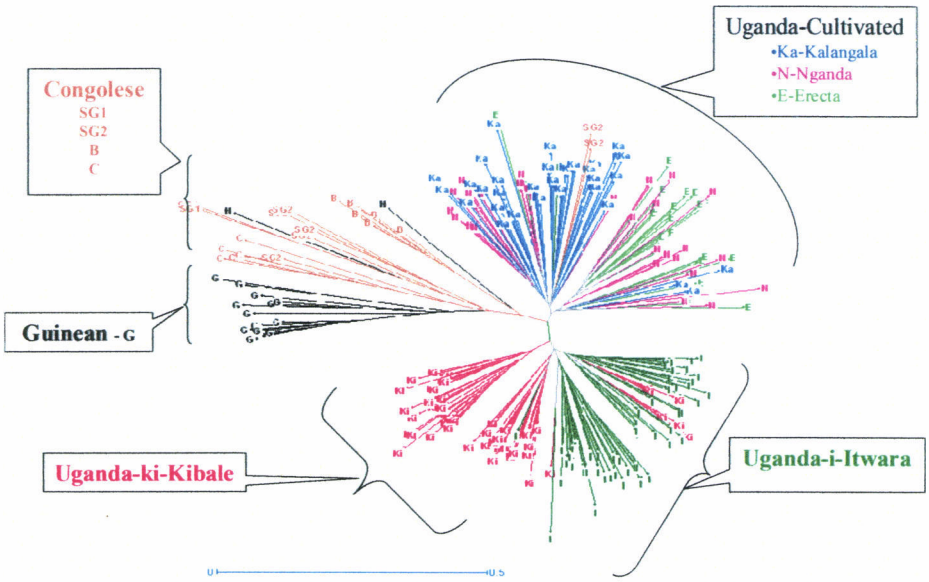


Figure 6: Dissimilarity tree of Ugandan C. canephora and other known diversity groups constructed using weighted neighbour joining method

3-7- Spatial and temporal spread of CWD

3-7-1 On-farm activities

Wilt incidence increased with time at all the farms but varied with farms probably due to different environmental conditions or field management at each farm. There was no evidence of variation due to age of coffee, inclination of farms (whether flat, level, or steep slope), pruning practices, cropping system, presence or absence of shade and how the diseased trees were managed.

3-7-2 On-station activities at Kituza

Disease progress curves showing temporal disease development and maps of diseased trees were generated from the disease data. Semi-variance analysis and kriging were performed on the data to show the spatial-temporal structure of disease. Host influence on the spatial-temporal structure was deduced through distribution pattern of diseased and healthy trees and analysis of variance. Results show that the disease epidemic progresses gradually overtime. The disease was found to spread irregularly from initial infections to healthy neighbour trees, leading to aggregated patterns. An infected tree can infect up to three healthy trees away, in any direction. Disease foci form and grow with time, coalescing to one continuous stretch, only punctuated in spots planted with resistant hosts. There were varying levels of susceptibility among host genotypes, exhibiting varying rates and levels of disease development.

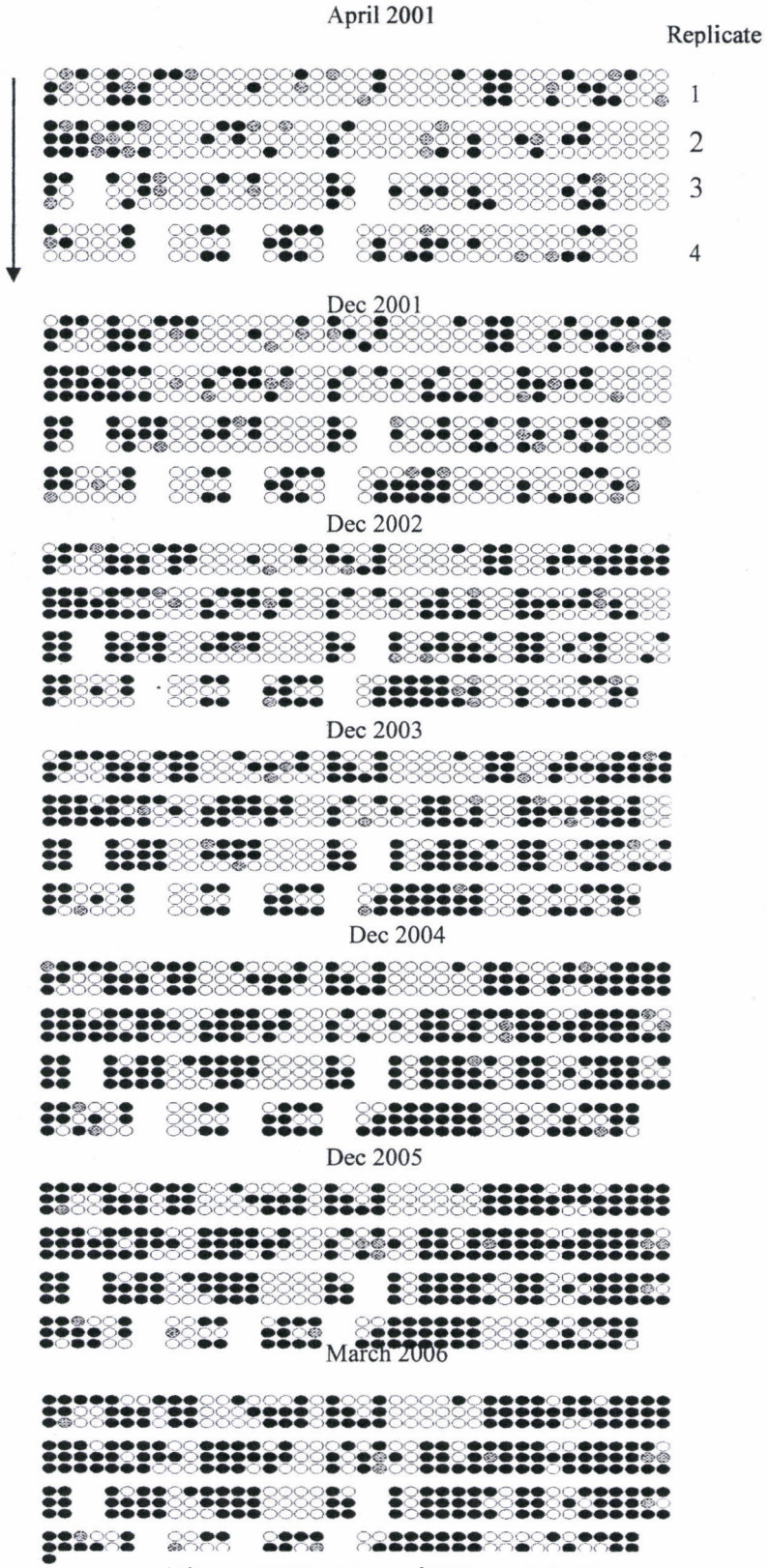


Figure 7: Pattern of diseased trees

Arrow is pointing down slope; Each tree is represented with a circle; Black circles are trees killed by CWD, Circle with cross squares show sick tree and white circles show uninfected trees; 4 replicates in the field separated by larger clear lines; 2 rowsx3 columns of circles in each replicate represent a clone; White gaps within replicates represent missing data; Trees uprooted prior to April 2001 are included among the dead.

3-8- - Longevity/survival of the CWD pathogen

3-8-1: Survival of *F. xylariodes* inoculum in the soils

Periodic planting of seedlings in specific *ex situ* soil samples infected with CWD to determine the longevity/survival of *F. xylarioides* inoculum in soil over time shows more seedlings were infected when planted in freshly sampled soil than in soil stored over a period of time from the three sites. Seedlings (3 out of 10) planted in freshly sampled forest soil developed wilt symptoms 76 – 83 days and died within 6 – 8 days from first symptoms. Subsequent monthly plantings in stored soil samples continued to reveal more wilting among plants in soil sampled from the forest and the wilting continued up to the 5th month of storage. This strongly implies that the pathogen can persist in soil and become source of inoculum. Since coffee nursery operators normally collect fertile soil from the forests, it is then advisable that they should sterilize the soil before use.

3-8-2. Role of farm tools/machete or panga in transmission of CWD

Different wounding of plants using infected machete demonstrated that there is possibility of transmission of CWD through contaminated tools commonly used on the farm. This therefore calls for sterilization of tools using alcohol, disinfectant or fire before it is used on healthy plants.

3-8-3. Survival of *F. xylarioides* in infected stem/wood pieces over time.

Repeated culturing of stored infected wood pieces continued to reveal presence of *F. xylarioides* for 6 months. No growth occurred on agar media from 7 – 12 months. Wood pieces therefore are likely to remain infective for at least 6 months from the death of trees, whether buried in soil or standing in the fields. Uprooting and burning where feasible is still recommended to reduce inoculum.

4- Problems encountered

The major problem encountered was delayed starting and completion of some activities within the project i.e. Part of the research work under this work package was supposed to be carried out at the Coffee Research Institute (CORI) in Uganda and some activities were supposed to be done at CIRAD in France. But most the *C. canephora* plant materials required for the work at CIRAD had to be imported from Uganda. This required a material transfer agreement between CIRAD and NARO, whose legal process was lengthy and that caused a delay of this activity by more than one year.

Secondly some activities, particularly the artificial pollinations (hybridization) are dependent on prevailing weather conditions. Dry weather or excessively wet conditions do not favour flowering. Extreme and prolonged dry season experienced between March and September 2004 did not allow flowering and consequently there was very limited artificial pollinations during this period. This caused a shortfall in the number of hybrid seedlings required for screen house studies on the inheritance of CWD resistance. Therefore there was a delay of this activity and consequently we requested for extension of the project by a year, to allow for this activity.

5-.Technology implementation plan

The results given above have very important practical perspective for developing CWD resistant *C. canephora* varieties and controlling the disease on this crop in Uganda using the variety resistance as indicated below.

5-1- Adoption of resistant clones identified in this study

Among the *C. canephora* clones evaluated in this study for field resistance to CWD, clones J/1/1 and Q/3/4 are resistant to this disease. The two clones can yield 3.3 and 2.7 metric tones (average) of clean coffee per hectare per annum, respectively and they are resistant to leaf rust and red blister diseases. Therefore these clones are currently being for validation in multi-location trials in different *C.*

canephora growing agro-ecological areas within the country, before they are released to farmers. The clones could be available to farmers in 6-8 years. Adoption of these clones will contribute to the control of coffee wilt disease and revitalizing Robusta coffee production in Uganda. But because of the out breeding nature of this crop, the two clones are too few for massive replanting required in the entire. .At least five different clones are recommendable for successful pollinations and fruiting and hence good yields. To avoid such risks, in the short run, the best of the moderately susceptible clones also studied in the field trials i.e. R/1/4 258s/24 and 1s/3 are also being multiplied for supply to farmers together with J/1/1 and Q/3/4, as we continue to develop more CWD resistant clones

5-2- Developing more CWD resistant varieties

The over 1500 CWD resistant individuals identified through large scale screening of using from different sources have been planted in mother gardens at CORI for vegetative propagation as clones. These clones shall be evaluation in on-station and on-farm field trials before superior clones are selected for release to farmers. New clones developed through this process are anticipated to be available in the next 18-20 years.

Meanwhile more resistant individuals can be identified through the artificial inoculations. The immediate source of plants for this analysis shall be mother plants of resistant half sib progenies sampled from Kalangala Island on Lake Victoria, Itwara and Kibale forests.

Therefore crossing specific CWD resistant clones with the current commercial clones has been initiated so as to combine the CWD resistance with high yields (2.5 tons of clean coffee per hectare per annum), good bean qualities (18-22g hundred beans weight, over 90% retained by screen 18/64) and good cup qualities of the commercial clones. It is anticipated that selection of new varieties through this process will take up 20 years.

5-3- Conservation of C. canephora genetic resources

Bearing in mind that CWD is indiscriminately destroying the Ugandan *C. canephora* gene pool plus the on going degradation of natural biodiversity reserves by human settlements and farming systems, it is important to protect this gene pool. Since genetic differentiation among *C. canephora* populations is high, samples of a few genotypes will be obtained from many populations to capture enough natural genetic variability for conservation and utilization in future breeding programs. Ugandan *C. canephora* samples will be collected from Itwara, Kalangala and sites in Kibale in addition to other relict forests and isolated cultivated regions not included in this study. Because of threats from coffee wilt disease and other unforeseen natural disasters on *C. canephora* genetic resources, efforts will be made to find new locations or collaborator for an international germplasm collection and conservation in multiple conservation centres in different countries to preserve.

6- Publications and papers

Conference papers

i) **Pascal Musoli**, Pauline Aluka, Philippe Cubry, Magali Dufour, Fabien de Bellis, James Ogwang, Denis Kyetere, Thierry Leroy, Daniel Bieysse, André Charrie. Fighting coffee wilt disease: Uganda wild *C. canephora* genetic diversity and its usefulness. ASIC 2006. 21st International Conference on Coffee Science, Montpellier, France

ii) Janzac B., **Musoli** P., Roussel V., Bonnemayre K., Pinnard F., Leroy T., Dufour M., Kyetere D., Hakiza G., Kalonji A., Tshilenge P., Girma A., Bieysee D. Control of coffee wilt disease: Study of genetic diversity of *Fusarium xylarioides* and *Coffea canephora* in Uganda. Poster paper on ASIC Conference, India, November 2004

iii) Hakiza, G.J, Birikunzira, J.B., Olal, S. and Kabole C. "Preliminary studies on the epidemiology of Coffee Wilt Disease (Gibberella xylarioides) in Uganda. Poster presentation. Proceedings of the 20th International Conference on Coffee Science, ASIC, Bangalore, India, October 11 – 15, 2004

iv) **Pascal Musoli**, Sam Olal, Charles Kabole, Agnes Nabaggala, 2002: Response of elite arabica coffee germplasm to *Fusarium xylarioides* infection in Uganda. **NARO conference**

Thesis

MUSOLI Chungason Pascal, 2007. Recherche de sources de résistance à la trachéomycose du caféier *Coffea canephora* Pierre, due à *Fusarium xylarioides* Steyaert en Ouganda. **Docteur en Sciences, de l'Ecole Nationale Superieure Agronomique de Montpellier**

Journal articles in preparation

Pascal Musoli, Philippe Cubry, Pauline Aluka, Claire Billot, Magali Dufour, Fabien De Bellis, Denis Kyetere, James Ochugo, Daniel Bieysse, André Charrier and Thierry Leroy. A new genetic diversity group from Uganda within *Coffea canephora* Pierre. Submitted to the Genetics Journal

Pascal C. Musoli, Christian Cilas, David Pot, Agnes Nabaggala, Saleh Nakendo, James Pande, André Charrier, Daniel Bieysse, Thierry Leroy. Inheritance of resistance to coffee wilt disease *(Fusarium xylarioides* Steyaert) in *Coffea canephora* Pierre. Submitted to New phytologist Journal

Pascal C. Musoli, C. Cilas, A. Charrier, A. Kangire, G.M. ten Hoopen, C. Kabole, J. Ogwang[1] and D. Bieysse. Spatial and temporal analysis of Coffee Wilt Disease caused by *Fusarium xylarioides* Steyaert in *Coffea canephora*. Submitted to European Journal of Phytopathology

7- Conclusion

The studies carry out in this project were successfully implemented and the results obtained are very pertinent to contribute to control coffee wilt disease in Uganda.

An artificial inoculation technique use for screening for resistance to CWD with a good correlation with the field resistance was developed. Until a better method is found root dip method will remain in use. Practical use of results in breeding for durable resistance where knowledge on strains of the pathogen is essential. Cultures are available for use in screening, and for various studies on the pathogene.

It is apparent from this study that disease spread within the field can occur from one infected plant to the next plant probably through the root contact. Dry wood in the field is the major source of inoculum, which forms abundantly in the form of perithecia. Human activities can accelerate disease spread within the field and beyond. This information can be used to restrict people from moving plant parts etc.

Many times farmers have asked how long the pathogen persists in soil, in infected wood pieces or if it can be transmitted through the use of common tools on the farm. This task has provided the information. The pathogen can persist in soil, wood for up to 6 months. Farmers are advised to uproot and burn infected plants. Soil for nursery use should be well sterilized and tools commonly used in the field should be sterilized by flaming or other means.

Information collected on the life cycle of the pathogen, is useful for formulation of control measures. We are aware that perithecia are a major source of inoculum, which forms on drying wood. This further strengthens the recommendation to burn all infected plants.

The screening protocols developed were used effectively in the identification of *C. canephora* clones which are resistant to coffee wilt disease. And the CWD resistant *C. canephora* clones identified in these studies shall be used either directly for re-planting or as breeding population for improving CWD resistance of the current commercial clones or as rootstock for grafting the commercial clones for re-planting in CWD infected areas to effectively control this disease.. This will take more time and need

financial resource to implement. Whether this evaluation then the right varieties shall not identified, thus reducing on the anticipated benefits of this work.

8- Acknowledgement.

This work was funded by the European Union. I wish to thank the Director of CORI and technicians, particularly A. Nabaggala, J. Pande, S. Nakendo and J. Ochugo for all their technical input in the work. I am indebted to Daniel Bieysse and Drs Thierry Leroy and Christian Cilas useful discussion and assistance in the analysis of various data.

INDIVIDUAL FINAL REPORT

PARTNER P4: Université de Kinshassa (UNIKIN)
République démocratique du Congo

Dr Adrien Kalonji
MsC Patrick Kalonji

1. Objectifs

Le laboratoire de Phytopathologie de la Faculté des Sciences Agronomiques de l'Université de Kinshasa a été partenaire dans le projet COWIDI financée par l'Union européenne sous le contrat numéro ICA4-CT-2001-10006.

L'objectif général du projet était de développer les stratégies nécessaires de lutte contre la trachéomycose du caféier canephora dans la zone préférentielle de culture , proposer des actions et des moyens susceptibles de contribuer à réduire le niveau de l'infection, ralentir l'expansion de la maladie et assurer une meilleure protection des zones caféières.

La trachéomycose est une affection causée par le champignon *Fusarium (Gibberella) xylarioides*. C'est une maladie non seulement dangereuse et grave pour les plantes déjà attaquées et irrémédiablement condamnées, mais également pour celles qui ne le sont pas encore et même pour des plantations éloignées. L'affection se manifeste avec brutalité en provoquant des destructions considérables des exploitations caféières dans les zones de production caféière de la RDC. Sa propagation s'effectue par les propagules (conidies ou ascospores) véhiculées par l'air, l'eau, les insectes. La contamination liée à l'activité humaine par des outils souillés, semble importante en particulier les infections par blessure lors des travaux d'entretien des caféières, ainsi que le transport de matériel végétal infecté. La présence des plantes malades dans les plantations, à défaut des moyens de lutte organisée, contribuerait à la propagation facile de la maladie. Réapparue sous une phase épidémique depuis les années 1982-1983 dans les plantations aussi bien paysannes qu'industrielles, elle est actuellement en RDC, la maladie qui cause le plus de dégâts aux caféiers.

Cette maladie réémergente avait été contrôlée lors de sa première expansion entre 1947 et 1960 par des dispositions pratiques et des mesures classiques de nature prophylactique, à savoir repérage systématique des plantes malades dès l'apparition des premiers signes, suivi de l'arrachage et de leur incinération sur place. La mise en application de ces mesures pour contrer l'extension de la trachéomycose s'étaient avérées efficaces, mais coûteux. Actuellement, leur mise en œuvre pour faire face à cette réémergence qui atteint des surfaces considérables n'est pas réaliste.

Les stratégies de recherche se sont orientées vers une lutte génétique. La recherche des matériels résistants (clones élites de l'INERA et autres matériels introduits) vis-à-vis de la trachéomycose et la prospection des clones présentant une résistance naturelle serviront dans le schéma futur de sélection par voie générative.
Les objectifs assignés au partenaire UNIKIN sont les suivants :

- Récolte des données relatives à la répartition de la trachéomycose
- Constitution de la collection des isolats à partir des matériels infectés et caractérisation des formes fusariennes associées au déperissement du caféier robusta
- Etude de la pathogénicité des isolats à travers les tests d'inoculations artificielles
- Evaluation de la résistance variétale
- Evolution spatio-temporelle de la trachéomycose et rôle des facteurs environnementaux et humains dans l'extension de la maladie
- Transfert des isolats et matériel végétal

2. Activités

2.1. Répartition de la trachéomycose

Les données relatives à la répartition de la trachéomycose en RDC ont été collectées sur terrain dans les plantations situées dans les provinces où la culture de caféier canephora est pratiquée à savoir Bas-Congo, Bandundu, Equateur, Kasaï-Occidental, Kasaï-Oriental, Nord-Kivu et Province orientale.. Les enquêtes ont été effectuées à partir d'un échantillon représentatif de 914 plantations; Les résultats se rapportent à l'état phytosanitaire des plantations, et au degré d'attaque des cultures par la trachéomycose.

2.2. Constitution de la collection de Fusarium xylarioides

On a procédé à la récolte des matériels pour la constitution d'une collection d'isolats de Fusarium xylarioides. Les isolements ont été faits à partir des échantillons collectés sur des caféiers atteints de la trachéomycose dans les zones de production caféière des provinces Orientale, Nord Kivu et Equateur.

2.3. Evaluation de l'agressivité des isolats

Les différentes souches de *F. xylarioides* isolées en RDC ont été testées pour évaluer leur agressivité. Des inoculations ont été faites sur des plantules sensibles de caféier robusta de génotype KR 12/5 âgées de 6 à 10 mois. La technique d'inoculation des plantules a été celle d'injection à l'aide de la seringue (Terumo, Myjector U-40 Insulin).

2.4. Caractérisation et évaluation de la diversité génétique des *Fusarium* spp.

On a isolé et caractérisé au point de vue morphologique et moléculaire en utilisant les marqueurs RAPD, différentes espèces de *Fusarium* associées au dépérissement du caféier robusta en RDC. Le but est d'établir la responsabilité de ces formes fusariennes dans le dépérissement du caféier.

2.5. Conservation du pouvoir infectieux et possibilité de transmission de la maladie

La présence d'un inoculum primaire dans un site figure parmi les facteurs les plus importants dans la propagation d'une maladie épidémique. Dans le cas de la trachéomycose du caféier une hypothèse a été émise sur la possibilité de contamination par différents débris parasités. Cet essai est entrepris en vue de déterminer la capacité de survie et de contamination de l'agent pathogène à des niveaux différents dans le bois parasité.

2.6. Effet des types de croisement (mating type) sur le développement de la trachéomycose dans les conditions naturelles

Les travaux récents conduits *in vitro* sur la sexualité de *G. xylarioides* avec des souches congolaises, ougandaises et tanzaniennes, ont démontré chez cette espèce que le mode hétérothallique est contrôlé par un locus avec deux allèles, Mating type 1 (MAT-1) et Mating type 2 (MAT-2). Les isolats compatibles produisent de périthèces fertiles, montrant le suintement des ascospores au niveau de l'ostiole, endéans 2 à 8 semaines après croisement. Nous avons étudié la sexualité de ces types de croisement dans les conditions naturelles et tenté de déterminer leur influence sur le développement de la trachéomycose par le biais de différents symptômes observés.

2.7. Détermination du niveau de résistance variétale

Les essais sur la résistance variétale ont été conduits à Beni (Nord-Kivu) en conditions naturelles d'expression de la trachéomycose. L'objectif poursuivi est le criblage de différents génotypes du caféier pour la résistance à la trachéomycose en vue de déterminer ceux qui présenteraient un bon niveau de résistance et qui peuvent être inclus dans le schéma de sélection pour leur diffusion auprès des planteurs sous forme des clones.

2.8. Variabilité génétique des accessions de caféier robusta

L'évaluation de la variabilité génétique dans les populations de *C. arabica* en utilisant des marqueurs moléculaires tels que RAPD, ISSR, ALFP, et SSR a fait l'objet de plusieurs études. Des analyses semblables ont été menées chez le *C. canephora*. pour évaluer le niveau de la variation génétique des accessions du café canephora de la RDC en utilisant des marqueurs moléculaires (RAPD et ISSR). Cette étude permettra d'évaluer la diversité génétique existante dans les collections et identifier des marqueurs corrélés avec la résistance à la trachéomycose.

Les accessions utilisées dans cet essai sont représentées par des embryons extraits des graines récoltées sur des pieds-mères plantés dans un Bloc ayant abrité un essai d'adaptation locale à Kiyaka de meilleures descendances clonales mis au point à Yangambi : SA 158, L 93, L 147, L 215 et L 251 (qui avaient subi avec succès l'épreuve de la descendance). Outre le matériel précité, l'essai d'adaptation locale entrepris à Kiyaka comprend un témoin SA 34 et deux autres descendances, L 36 et L 48. L'introduction de ces clones à Kiyaka a été faite au moyen des graines récoltées sur ces clones et mises en germination à Kiyaka en 1954. La mise en place définitive avait eu lieu le 7 décembre 1954. La récolte des graines ayant constitué l'échantillon de la présente étude a été effectuée sur de différents pieds-mères de différentes lignes dans ce Bloc.

2.9. Transfert de matériel et isolats

Des échantillons des caféiers malades et des semences récoltées en RDC sur des sujets intéressants du point de vue productivité dans les plantations de l'Institut National pour la Recherche et l'Etude Agronomique (INERA) de Luki (Bas-Congo) et de Kiyaka (Bandundu) ont été envoyés au CIRAD (Montpellier) et à l'Université catholique de Louvain (UCL). Des accessions étrangères introduites provenant de la Côte d'Ivoire ont été ajoutées. Les génotypes ont été de type Kouillou et Robusta.

2.10.Formation

L'UNIKIN a bénéficié d'un financement pour la formation de M. Patrick Tshilenge dans le cadre d'un DEA en phytopathologie à l'UCL durant l'année universitaire 2003-2004.

2.11. Publication

Une présentation des résultats a été réalisée dans les rapports annuels du présent Projet et lors de présentations dans des congrès nationaux ou internationaux des acteurs de la filière.

3. Résultats

3.1. Répartition et évolution de la trachéomycose

Les résultats des enquêtes phytosanitaires ont permis de localiser la dispersion de la trachéomycose en RDC. Sa présence est signalée dans les provinces de l'Equateur, du Nord-Kivu et Orientale. Sa proportion globale représente environ 35 % soit 323 plantations sur 914 plantations inspectées. Les plantations des provinces du Bas Congo, Bandundu, Kasaï Occidental et Kasaï Oriental n'ont pas révélé la présence de cette maladie.

L'analyse de ces données montre, pour les provinces Orientale et du Nord-Kivu, que les attaques de la trachéomycose se sont intensifiées et ont connu dans le temps une évolution de type logarithmique jusqu'en 2002 et atteint des valeurs qui oscillent entre 82,6 % (Poko) à 100 % d'incidence (Isiro, Muhangi et Bafwasende). A Yangambi, dans la Province Orientale une situation particulière est à signaler ; la trachéomycose qui a été absente jusqu'à 1996 est apparue avec une incidence très élevée (81,8 %) et se retrouve dans toutes les plantations de ce Centre de Recherche (100%). Elle accuse une tendance à la baisse à partir de 2004. Pour la province de l'Equateur les données indiquent que la présence de la trachéomycose a été signalée dans le district de la Mongala de part frontière avec la province Orientale où elle sévit déjà. La maladie évolue de la province Orientale vers celle de l'Equateur.

3.2. Constitution de la collection de Fusarium xylarioides

A partir des échantillons malades, une collection des souches monoconidiennes de *F. xylarioides* et d'autres espèces fusariennes isolées de ces échantillons a été constituée. Certains isolats sont conservés dans la Mycothèque de l'Université catholique de Louvain (MUCL).

Tableau 1 : Liste de souches de la collection l'Université de Kinshasa (UNIKIN)

N° d'ordre	Espèce	Souche	N° MUCL	Origine en RDC	Date de récolte par [1]	Date d'isolement par [2]	Identifié par [3]
01	*F. equiseti*	PTFsp4	46054	Isiro	17/12/02 K.T.D.	16/01/03 T.	T. & FM.
02	*F. falciforme*	MUCL43880	43880	Bas-Congo	12/03/02 K.T.D.	17/03/02 HM.	HM.& FM.
03	*F. solani*	PTFsp1	-	Isiro	17/12/02 K.T.D.	16/01/03 T.	T. & FM.
04		PTFsp2	46055	Yangambi	15/12/02 K.T.D.	16/01/03 T.	T. & FM.
05		PTFsp5	-	Isiro	17/12/02 K.T.D.	16/01/03 T.	T. & FM.
06		SR21/03	45431	ButemboNjiya	12/09/02 NV.	17/12/02 L.	L.
07	*F. stilboides*	SR17/09	45428	ButemboNjiya	12/09/02 NV.	17/12/02 L.	L.
08	*F. xylarioides*	Abdoul	46043	Isiro	15/09/03 K.D.	16/10/03 T.	T.
09		B10101(2)J	46044	Beni-Mutwanga	02/12/02 K.T.D.	02/01/03 T.	T.
10		B10101(5)	46045	Beni	02/12/02 K.T.D.	02/01/03 T.	T.
11		B103	46046	Beni-Mangina	28/08/03 K.D.	16/10/03 T.	T.
12		B20301(3)	46047	Beni	02/12/02 K.T.D.	02/01/03 T.	T.
13		B30301(3)	46048	Beni	02/12/02 K.T.D.	02/01/03 T.	T.
14		IPMO2(2)G	46049	Isiro	17/12/02 K.T.D.	02/01/03 T.	T.
15		Kambale	46050	Beni-Oicha	08/09/03 K.D.	02/01/03 T.	T.
16		Lebruniana	46051	Yangambi	15/12/02 K.T.D.	02/01/03 T.	T.
17		Mayimoya	46052	Beni	09/09/03 K.D.	16/10/03 T.	T.
18		MUCL 14186	14186	Yangambi	1960 Meyer	Meyer	Meyer
19		MUCL35223	35223	Isiro	Nov. 1992 P. Decock	1992 Decock	Decock
20		SR01A/05a	44516	Beni	02/12/02 K.T.	17/12/02 L.	L.

				D.	L.	
21	SR01B/10a	45580	Yangambi (L147)	15/12/02 K.T. D.	21/05/03 L.	L.
22	SR12B/01a	45592	Isiro	17/12/02 K.T.D.	21/05/03 L.	L.
23	SR22/01a	44512	Butembo	12/9/02 NV.	17/12/02 L.	L.
24	Twaze	46053	Isiro	15/09/03 K. D.	16/10/03 T.	T.
25	14/SS02-MAT1*	_				L.
26	15/SS05-MAT2*	_				L.
27	Bandazwa	47046	Itimbiri	09/09/2004 K.D.	20/11/2004	T.
28	Bunduki	47051	Proche Oriental	09/09/2004 K.D.	20/11/2004	T.
29	Isangi	47050	Lisala	17/09/2004 K.D.	20/11/2004	T.
30	Kermu		Basankusu	15/10/2004 K.D.	20/11/2004	T.
31	Lisanza Mahonde		Loeka	15/09/2004 K.D.	20/11/2004	T.
32	Mabanda	47054	Itimbiri	09/09/2004 K.D.	22/11/2004	T.
33	Maleka Maluku	47045		15/09/2004 K.D.	22/11/2004	T.
34	Mangbakapale	47056		15/09/2004 K.D.	20/11/2004	T.
35	Mindembo Bloc 5	47053	Lisala	17/09/2004 K.D.	20/11/2004	T.
36	Mindembo Bloc 17	47047	Lisala	17/09/2004 K.D.	22/11/2004	T.
37	Moboko	47055	Axe Itimbiri	09/09/2004 K.D.	22/11/2004	T.
38	Mongene	47041	Itimbiri	10/10/2004 K.D.	22/11/2004	T.
39	Ngwa	47042	Itimbiri	10/09/2004 K.D.	22/11/2004	T.
40	N.-Dame des Pauvres	47039	Loeka	15/09/2004 K.D.	22/11/2004	T.
41	Oscar Adongo	47057	Loeka	14/09/2004 K.D.	22/11/2004	T.
42	Payipayi Mabanda	47052		15/09/2004 K.D.	22/11/2004	T.
43	Vil basalaka	47040	Itimbiri	11/09/2004 K.D.	22/11/2004	T.
44	Yabia	47048	Loeka	15/09/2004 K.D.	20/11/2004	T.
45	Yanangi	47058	Itimbiri	09/09/2004 K.D.	20/11/2004	T.
46	Yangoy/Basalak	47044	Itimbiri	11/09/2004 K.D.	20/11/2004	T.
47	Yeboka	47043	Itimbiri	09/09/2004 K.D.	20/11/2004	T.

48	Zobolia	47049	Loeka	15/09/2004 K.D.	20/11/2004	T.
49	JE Mamba		Jardin Expér.	13/04/2005 T.	13/04/2005	T.
50	Otomba lodja		K.Oriental/lodja	24/12/2005		
51	Shilo lodja		K.Oriental/ lodja	24/12/2005		
52	Betonge		Province orientale			

3.3. Evaluation de l'agressivité des isolats

Les résultats obtenus ont montré une grande variabilité dans le pouvoir pathogène des souches testées. Cette variabilité, comparée à la faible diversité obtenue par l'analyse moléculaire, suggère que l'agressivité chez le *F. xylarioides* pourrait être un caractère fluctuant en fonction des conditions externes tans du milieu qu'expérimentales. L'intérêt d'une telle variabilité résiderait dans le choix des souches les plus contrastées lors des tests de résistance dans de l'interaction hôte-pathogène

3.4. Caractérisation et évaluation de la diversité génétique des *Fusarium* spp.

L'isolement et la caractérisation des différentes espèces de *Fusarium* associées au dépérissement du caféier robusta a montré pour des caractères culturaux, des différences entre les souches du *Fusarium xylarioides* et celles des *F.equiseti, F.falciforme, F.solani* et *F. stilboides*. Les inoculations ont révélé que seules les souches de *F.xylarioides* induisent la maladie et la mortalité des plants. Il n'a pas été établi la responsabilité des autres formes fusariennes. L'analyse de la diversité à l'aide des marqueurs RAPD a permis de révéler une nette différence entre les différentes espèces de *Fusarium*. Par contre au sein des souches de *F. xylarioides*, aucune diversité n'a été observée avec les amorces utilisées.

3.5. Conservation du pouvoir infectieux et possibilité de transmission de la maladie

L'analyse des données montre que l'induction des symptômes de la maladie a été observée avec tous les types d'inoculum. Par contre la mortalité a été enregistrée uniquement sur les plants inoculés à l'aide des tissus immédiatement sous écorce et le fragment de culture gélosée du parasite. L'absence de cette manifestation chez les sujets inoculés avec les écorces portant périthèces serait être due à l'identité de ces périthèces qui seraient autres que ceux de *G. xylarioides*, capables pourtant de provoquer des symptômes courants sauf la mortalité. Une telle observation a été enregistrée lors des inoculations faites avec le *F. solani, F. stilboides* et *F. falciforme* (Tshilenge *et al.*, 2004). Les tissus profonds qui n'ont pas également provoqué la mortalité pourraient être considérés comme hors d'atteinte du parasite et par conséquent être non contaminants.

3.6. Effet des types de croisement (mating type) sur le développement de la trachéomycose dans les conditions naturelles

L'étude des isolats de mating type opposés, inoculés sur la même plante ou séparément ont permis de déterminer l'ordre chronologique de l'apparition des manifestations symptômatologiques suivantes : le brunissement des feuilles, le dessèchement des feuilles et la mortalité des plants. Le moment d'apparition de ces différents symptômes varie selon les traitements.

La période d'incubation, conduisant à l'apparition du brunissement n'est pas différente quel que soit le traitement (p=0.660). Une nette différence existe entre le niveau de brunissement des feuilles des plants non inoculés (Témoin) et celles inoculés. La comparaison de 2 types de croisement, utilisés isolément en inoculation ou en combinaisons ne révèle pas des différences dans la sévérité de brunissement des feuilles.

Les mating types, utilisés seuls ou en combinaison présentent des cas de dessèchement des feuilles, contrairement aux plants non inoculés (p=0.02296). Ils montrent aussi des différences en ce qui concerne le dessèchement des feuilles (p=5.817e-06) avec les plants soumis au Mating Type 1 qui se

dessèchent plus tardivement que les autres (p=0.043). Cependant les plants inoculés avec le MAT-1 semblent avoir moins de feuilles desséchées que ceux inoculés avec le MAT-2 ainsi que dans tous les cas où ce dernier intervient en combinaison. L'influence de MAT-2 est encore prépondérante comme dans le cas de taux brunissement des feuilles. En admettant le seuil de 20%, cet effet retard de MAT - 1 dans l'induction de ce symptôme pourrait être perçu et hiérarchisé dans quelques-unes de ses combinaisons comme suit : MAT-2 sur MAT - 1 (p=0.06), MAT - 1 face MAT - 2 (p=0.18) et MAT - 1 sur MAT - 2 (p=0.20).

La mortalité intervient à des périodes différentes (p=1.199e-12) selon le type de croisement. L'inoculation avec le MAT - 1 induit plus tardivement la mortalité. Dans la combinaison, l'influence de MAT - 2 est plus importante quelle que soit la position d'inoculation. seuls les plants inoculés avec les 2 types de croisements ont connu de manière très significative (p= 0.002982) des cas de mortalité, contrairement ceux qui ne sont pas inoculés. Le Mating type 1 semble avoir une faible importance dans la mortalité des plants, tandis que le Mating type 2 aurait un effet contraire, seul ou dans toutes ses combinaisons où il intervient.

A l'issue des observations sur le moment d'apparition et la sévérité des symptômes, il convient de retenir l'effet plus marqué de Mating type 2, inoculé seul et en combinaison avec le Mating type 1. En effet, lorsque des différences sont enregistrées entre les deux types de croisement, le Mating type 2 entraîne plus précocement le dessèchement des feuilles et la mortalité des plants que le Mating type 1. Il en est de même dans leurs combinaisons où la tendance est orientée vers le Mating type 2.

Les corrélations calculées révèlent des différentes valeurs entre les symptômes observés. Une corrélation positive a été trouvée entre le dessèchement et mortalité (R^2 = 0.8304). Par contre entre le brunissement et la mortalité, il n'y a pas eu de corrélation (R^2 = 0.2212), comme entre le brunissement et le dessèchement (R^2 = 0.1174).

Les périthèces sont plus abondants sur les plants inoculés avec le Mating type 2 (cote 2) que sur les inoculés avec le Mating 1 (cote 0). En combinaison, aucune tendance dans la position de l'un par rapport à l'autre n'est perceptible.

Tableau : Production des périthèces enregistrée après inoculation avec les Mating types 1 et 2 en différentes positions sur la tige des plantules de caféiers.

Position des points d'inoculation	Abondance	Fréquence (%) de différents états		
		S	G	E
MAT - 1	0	0	0	0
Mat 2	2	20	0	0
MAT - 1 face à MAT - 2	2	50	12,5	0
MAT - 1 sur Mat 2	3	33,3	16,6	0
MAT - 2 sur Mat1	3	16,6	0	16,6
Témoin	0	0	0	0

L'état de périthèces bien formés (état G) est enregistré avec des combinaisons de deux types en position face-à-face (12,5 %) et lorsque le Mat–1 est au-dessus de Mat–2 (16,6%). Par contre sur les plantules inoculées avec le Mat-1 et tout comme sur celles qui n'ont pas été inoculées, aucun état de périthèce n'est enregistré.

3.7. Détermination du niveau de résistance variétale

On a observé au regard du pourcentage de mortalité enregistré des différences ($p<0,05$) dans le comportement des génotypes de caféier utilisés pour ce qui est de leur résistance à la trachéomycose. De différents groupes qui sont dégagés par le test de Tukey sont présentés dans le tableau suivant.

Tableau : Comportement variétal (% de mortalité de plants inoculés) de différents génotypes de caféier testés pour la résistance à la trachéomycose.

N° d'ordre	Génotype	% de plants morts	Groupe
1	KR16/13A	6,6329	**a**
2	KR19/1B	7,5329	**a**
3	KR8/10	7,5329	**a**
4	KR19/11	7,6329	**a**
5	KR20/51	7,7829	**a b**
6	KR19/18B	8,0829	**a b**
7	KR17/55	8,1829	**a b**
8	KR10/7A	8,3829	**a b**
9	KR18/10	8,4329	**a b**
10	KR19/28	8,9329	**a b c**
11	KR19/26	9,0829	**a b c**
12	KR6/6	9,2829	**a b c**
13	KR18/30	9,3329	**a b c**
14	KR19/1A	9,5329	**a b c**
15	KR19/55	9,6829	**a b c**
16	KR20/50	10,1829	**a b c**
17	KR19/31	10,3329	**a b c**
18	KRA/6	10,5829	**a b c**
19	KR16/55	11,0329	**a b c**
20	KR17/47	11,1829	**a b c**
21	KR1/3	11,2329	**a b c**
22	KR16/13B	11,2329	**a b c**
23	KR18/10A	11,3829	**a b c**
24	KR1/1	11,5329	**a b c**
25	KR19/18A	11,8329	**a b c**
26	KR10/7B	12,0829	**a b c**
27	KR19/12	12,3329	**a b c**
28	KR20/31	12,3829	**a b c**
29	KRC/3	12,4829	**a b c**
30	KR3/5	12,6329	**a b c**
31	KR12/6A	12,8829	**a b c**
32	KR8/8	13,2829	**b c**
33	KR20/10	14,2829	**b c**
34	KR9/8	14,9829	**b c**
35	KR2/5	16,0829	**b c**

Les génotypes étudiés se classent en 4 différents groupes représentés par des lettres différentes. Le premier comprend les génotypes avec le pourcentage de mortalité des plants allant de 6,63 à 7,63 % ; le deuxième de 7,78 à 8,43 %; le troisième de 8,93 à 12,88 % et le quatrième de 13,28 à 16,08 %. Le premier et le quatrième sont les plus contrastés et peuvent suggérer, pour le présent essai, le seuil de 8 % comme seuil supérieur des génotypes résistants et celui de 13 % comme seuil inférieur des génotypes sensibles. Les génotypes avec des valeurs intermédiaires peuvent encore subir des tests de confirmation de la résistance.

3.7. Variabilité génétique des accessions de caféier robusta

Six amorces RAPD et 7 amorces ISSR, synthétisées par Invitrogen Life Technologies. Des ces deux types d'amorces utilisées, les RAPD ont détecté un niveau de polymorphisme plus élevé (95%) que les marqueurs d'ISSR (52%).

Amorces	Séquences des amorces (5' – 3')	Nombre de fragments amplifiés	Taille des fragments amplifiés	Nombre de fragments polymorphiques
Amorces RAPD				
UBC 186	GTGCGTCGCT	27	200-2000	27
Grasse 2	GTGGTCCGCA	8	150-1200	8
OPA 4	AATCGGGCTG	0	0	0
OPA11	CAATCGCCGT	13	300-2000	13
OPH 20	GGGAGACATC	0	0	0
Grasse 6	CGTCGCCCAT	9	300-1200	6
E 12	TTATCGCCCC	0	0	0
Amorces ISSR				
Echt 5	AGACAGACGC	5	250-1000	2
HB 13	GAGGAGGAGGC	8	250-800	7
HB 15	GTGGTGGTGGC	8	300-1000	2
UBC 825	ACACACACACACACACT	7	450-1100	7
SC ISSR 1	(AG) 8RG	7	250-850	0
17899A	CACACACACACAAG	9	250-1400	5

Les deux types de marqueurs étaient efficaces dans la détection de la variabilité génétique des accessions analysées. Cette grande variabilité génétique serait en partie due à l'allogamie de *C. robusta,* mais elle serait aussi attribuable à une grande différence génétique des clones qui ont constitué le patrimoine héréditaire initial. En fait les descendances examinées sont les produits des générations de quelques intercroisements de 15 différentes familles issues de lignées ayant des liens maternels. Malgré cette observation, les résultats enregistrés n'ont montré aucune tendance à la dérive génétique dans ces populations.

L'intérêt de tels résultats résiderait dans la sélection et amélioration génétique pour des caractères comme la résistance à la trachéomycose. En effet, 14 accessions de cette collection ont été testées pour la résistance à la trachéomycose. Elles ont montré à des degrés divers un certain niveau de résistance à cette maladie. Ceci indique qu'elles peuvent être utiles dans des programmes d'amélioration, non seulement pour l'accroissement de rendement pour lequel ils ont été initialement sélectionnés, mais également pour la résistance à la trachéomycose en RD Congo.

Ces résultats concordent avec ceux observés par Prakash *et al.*, (2005) en Inde en comparaison avec des accessions provenant du centre de la diversité génétique du café en Afrique en utilisant des marqueurs de SSR et d'AFLP. D'autres comme Aga *et al.*, (2005) ont quant a eux trouvé des niveaux de polymorphisme assez bas (25%) dans le matériel génétique de *Coffea arabica* d'Ethiopie à l'aide des amorces d'ISSR comparé à 52 % de polymorphisme observé dans notre cas. Le niveau de la variabilité génétique bas chez *C. arabica* rapporté dans plusieurs études pourrait être attribué en partie à sa nature autogame (Chaparro *et al.*, 2004).

Les analyses de RAPD ont montré également de plus grandes distances génétiques parmi les accessions étudiées que les données d'ISSR. Les différences entre les variations détectées par les amorces de RAPD et celles d'ISSR résultent de différentes régions génomiques visées. Les marqueurs de RAPD indiquent le polymorphisme dans les régions codantes et non codantes, y compris des séquences répétées ou des séquences uniques qui couvrent le génome entier

En conclusion, les marqueurs de RAPD ont semblé être plus informatifs que des marqueurs d'ISSR pour la caractérisation, l'analyse et l'utilisation des ressources génétiques de café robusta. Les deux systèmes sont basés sur l'application de PCR et ils sont faciles à appliquer a une analyse a grande échelle de matériels dans un programme d'amélioration génétique.

Pour une évaluation efficace de la variabilité génétique de ressources génétiques, ces deux types de marqueurs moléculaires devraient être utilisés conjointement pour couvrir une plus grande partie du génome. Dans la présente étude, le niveau de polymorphisme détecté était très élevé bien que les accessions aient été dérivées de familles ayant des liens maternels assez étroits. Eu égard à cette variabilité génétique, au niveau appréciable de la résistance à la trachéomycose ainsi qu'à la productivité individuelle, ces accessions ont un grand potentiel dans l'amélioration de Caféier robusta pour la résistance aux maladies et pour un rendement élevée. Leur distribution au niveau des planteurs serait recommandée pour la régénération de plantations de caféier robusta qui sont dégénérées par l'introduction des gènes venant d'autres accessions locales à faible rendement et faible résistance aux maladies.

3.8. Transfert de matériel et isolats

Les listes de matériels et isolats transférés aux différents partenaires existent au CIRAD à Montpellier et à l'UCL à Louvain. Les génotypes ont été de type Kouillou et Robusta.

3.9.Formation

Monsieur Patrick Tshilenge a complété sa formation en suivant un programme de Diplôme Etudes Approfondies (DEA) à l'UCL (13/09/2003-17/10/2004) orienté sur l'étude sur les différentes espèces fusariennes dans la pathogenèse de la trachéomycose ayant comme titre : "Caractérisation des *Fusarium* spp. associées au dépérissement du caféier robusta en République Démocratique du Congo". Il est actuellement inscrit au Doctorat à l'Université de Kinshasa et la soutenance de sa thèse est prévue pour la période fin juillet début août 2007.

3.10. Publication

A la suite des résultats obtenus, un article a été publié dans la revue Parasitica, 2004, volume 60 (3-4) pp 67-82. Il est intitulé " Caractérisation des *Fusarium* spp. associées au dépérissement du caféier robusta en République Démocratique du Congo."

L'équipe de l'UNIKIN a participé aux différents meetings organisés dans le cadre de d'échange sur les différents aspect de la caféiculture :

1. 4th African fine coffee Conference and exhibition, 15-17 february 2007. Addis Ababa, Ethiopia.
2. Final meeting on INCO Projects Coffee Wilt Disease (COWIDI), 9-10 septembre 2006, Montpellier (France).
3. Association Scientifique International du Café (ASSIC) '21st International Conférence on Coffee Science. 11-15 september 2006- Montpellier- France.
4. Regional Coffee Wilt Programme- Annual Review & Planning Meeting : Mainstreaming the Prevention & Management of Coffee Wilt Disease in Government Policy in Africa. 8th- 9th December 2005, CABI-ARC, Nairobi, Kenya.
5. Training course sur l'identification des espèces fusariennes rencontrées sur le caféier. CABI, mars 2003, Kampala, Uganda.
6. First meeting on INCO Projects Coffee Wilt Disease (COWIDI), 4-7 mars 2002, Kampala, Uganda.

Par ailleurs, l'UNIKIN a participé des meetings au niveau local, organisés régulièrement avec différentes Associations des planteurs de café dans le but de diffuser les résultats acquis.
Les principales Associations concernées sont :

A . Pour le Nord-Kivu :
- Coopérative des Producteurs du Café et Vivres (COOPROCAVI), à Beni au Nord-Kivu ;
- Syndicat pour Défense des Intérêts des Paysans (SYDIP) : Nord-Kivu ;
- Coopérative pour la Commercialisation des Produits du Nord-Kivu (COCOPNOKI) ;
- Association Paysanne pour le Developpement (APADER) à Muenda :
- Lide de Développment (LIDE).

B. Pour le district de Haut –Uélé (Isiro)
- Association des Agronomes pour le Développement de 'Uélé (ASSADU)

4 Conclusion

Les nouvelles connaissances acquises ont permis de mettre très clairement en évidence *Fusarium xylarioides* comme seul agent causal de la trachéomycose, la présence d'autres espèces de *Fusarium*, comme, *F. solani, F. stiboides, F. equiseti et F. falciforme,* étant présent comme pathogènes opportunistes.

Le développement d'un test d'inoculation artificielle a permis l'identification de matériel résistant en cours de multiplication, qui va être installé dans différents contextes agroécologiques. Par ailleurs, les premiers résultats de l'analyse de la diversité génétique à l'aide des marqueurs RAPD des caféiers en collection a mis en évidence la diversité des ressources génétiques des caféiers disponibles en collection. Ces caféiers constituent un réservoir de gènes pour l'amélioration vis-à-vis de la résistance à la trachéomycose mais aussi pour d'autres caractères agronomiques.

Le projet a montré la nécessité de collaborer étroitement entre partenaires du Sud et du Nord et la complémentarité des approches et constitue une première étape pour des collaborations futures.

INDIVIDUAL FINAL REPORT

PARTNER P4: CABI Bioscience (E-UK)

Dr Mike Rutherford

Introduction

Efforts to effectively manage CWD have been hindered by our lack of knowledge of the pathogen, *G. xylarioides*, and how it behaves. Specifically, it is important to understand whether variability exists within the pathogen population from a morphological, genetic, biological and pathogenic perspective to enable us to know precisely what we are dealing with and to support the development and introduction of a suitable management strategy. This is particularly important where quarantine measures and the use of host resistance are considered to be major and long term components of the strategy, as is the case with CWD. During the course of this project a range of scientific approaches were employed by CABI to assess, as part of Work Package 1, the extent of variability that exists within the CWD pathogen. The following provides an overview of the main areas of research undertaken and the key findings. For the most part these proved very effective in providing new knowledge.

1. Objectives

The overall objective of the research was to improve our knowledge of the coffee wilt pathogen, *Gibberella xylarioides* (presumed anamorph *Fusarium xylarioides*) with respect to genetic diversity. To achieve this objective a number of specific activities were undertaken under Work Package 1 in collaboration with project partners in Africa and Europe. These were intended to:

- Establish a collection of the anamorph (*F. xylarioides)* and teleomorph (*G. xylarioides)* forms of the CWD pathogen obtained from wilt-affected coffee trees in Uganda, Democratic Republic of Congo (DRC) and other regions of Africa as appropriate. Deposit representative strains in a designated facility to facilitate secure, long-term storage.
- Investigate the extent of diversity among strains, across geographical locations and over time, principally using a range of genetic approaches.
- Establish and maintain baseline data relating to those strains acquired and held by CABI and its partners on an electronic database.
- Synthesise results of research activities undertaken by project partners contributing to Work Package 1.

2. Activities

2.1 Establishing a collection of anamorph and teleomorph forms of the CWD pathogen

To support the research activities undertaken by CABI and by the other project partners, a collection of purified strains of the anamorph (*F. xylarioides*) and teleomorph of *G. xylarioides* and other fusaria from coffee and a number of other plant hosts was established at the CABI E-UK Centre (Egham, UK). The strains were obtained from coffee wood pieces collected by project scientists during the project and also donated, on request, from project partners in France, Belgium, Uganda and Democratic Republic of Congo (DRC), other research organisations and recognised culture collections around the world (including stocks already held at CABI). All strains, irrespective of origin, were purified at CABI by single spore isolation prior to inclusion in research activities or onward donation to partners. Where possible, the identity of purified strains was determined to species level on synthetic nutrient agar (SNA) and Potato Sucrose Agar (PSA) based on established morphological charaters.

Strains were maintained over the short to medium term on SNA slopes in darkness at 5°C and subcultured on fresh SNA plates at 25 °C for a minimum of 5 days prior to inclusion in the various experimental studies described below. Sixty strains, considered to be representative of the range of species obtained, their geographic origin and host of origin (species, variety or clone), were selected from the collection as a representive subset for in-depth study of variability using a number of approaches as described below. Seventy strains, including members of the representive group, were

deposited for long term storage under liquid nitrogen and in a freeze-dried state in the Genetic Resources Collection at CABI E-UK.

2.2 Investigate diversity among strains, across geographical locations and over time, principally using a range of genetic approaches.

Molecular variability

Genetic variability within the representative subset of strains, including *G. xylarioides*, *F. solani*, *F. stilboides*, *F. lateritium* and *F. oxysporum*, was evaluated by a range of primarily PCR based molecular approaches. This was a comprehensive study, the main objectives being to (i) to investigate genetic variation at and below species level (ii) identify any population structure existing within the CWD pathogen (iii) determine whether individual *G. xylarioides* strains could be demarcated as a basis for monitoring in epidemiology studies (iv) discriminate between strains of *G. xylarioides* and those of the other fusaria found on coffee.

The molecular approaches applied were chosen due to their fundamentally distinct approaches and to provide an opportunity to reveal the occurrence of any artefactual results. They enabled a study not only of the entire fungal genome, but also of specific regions of the genome (e.g. ribosomal intergenic spacer [IGS] region) and of DNA located in different areas of the fungal cells (e.g mitochondria), and would help to ascertain if, for example, a new form of the pathogen had emerged and become prevalent during the current CWD outbreak. Differences in IGS sequence, for example, have previously been used to delimit special forms of *F. oxysporum*. ISSR-PCR, which can be discriminatory at inter- and even intra-specific level in fungi may be employed to reveal genetic variability among fungi in a similar manner to RAPDs, but is deemed to provide more accurate discrimination among individual strains.

All strains for DNA analysis were grown for five days at 27°C in 60 ml liquid Glucose Yeast Medium (GYM), and cultures subsequently harvested by vacuum filtration and freeze dried. Freeze dried mycelium was disrupted using a pestle and mortar and total DNA extracted for analysis.

Vegetative compatibility group testing

Vegetative Compatibility Groups (VCG) have frequently been used to identify and discriminate genetic populations within a broad range of *Fusarium* species, including *F. oxysporum* where more than 20 VCG have now been identified. In some instances (e.g. *F. oxysporum* f. sp. *cubense*) strong correlation has been found between VCG and other genetic markers, including mitochondrial and RFLP profiles. This is indicative of the existence of clonally reproducing populations that may, for example, have become geographically isolated or have a strong relationship with a specific host. VCG determination involves the generation and pairing of nitrate non-utilising mutants. Where potentially compatible mutants complement one another for nitrate utilisation, they are considered to share identical alleles at loci governing incompatibility and therefore to be vegetatively compatible. Attempts to identify VCG within 16 of the representative subset of strains were made in the early stages of the project.

2.3 Establishment and maintenance of baseline data relating to those strains acquired

An electronic database, based on Excel software, was established at CABI E-UK to facilitate collation and storage of data relating to the various fusarium strains obtained and studied by the project partners.

3. Results

3.1 Establishing a collection of anamorph and teleomorph forms of the CWD pathogen

An extensive collection of more than 300 purified (monosporic) strains was established at CABI E-UK Centre. The majority were identified as *G. xylarioides* and isolated from *Coffea arabica* cultivated in Ethiopia and *Coffea canephora* cultivated in Uganda, DRC and Tanzania, from plants affected since an apparent re-emergence of the disease in DRC (1986) and subsequently Uganda (1993) and Tanzania (1997). A small number of strains, originally obtained from *C. canephora* affected by CWD in DRC, Central African Republic (CAR), Guinea and Ivory Coast (IC) in the 1950s and 1960s (i.e. during the earlier and very serious outbreaks in these countries) were also obtained from a number of established collections, including that of CABI E-UK. Strains of *Fusarium stilboides*, *Fusarium lateritium*, *Fusarium*

solani and *Fusarium oxysporum*, other recognised pathogens of coffee, were also obtained and included in research studies for comparative purposes. Of note, several strains received at CABI as the anamorph of *G. xylarioides* were shown, through morphological examination, to belong to other fusaria (namely *F. solani*, *F. stilboides* and *F. lateritium*) or to have been mixed cultures. As the morphological designation to species level were supported by genetic profiles (approaches described below), this not only emphasises the need for accurate morphological identification to support field diagnoses and characterisation studies, but also highlights the benefits of the genetic techniques selected and employed during the project. The culture collection provided an invaluable reference resource for the project and will be maintained for the benefit of future research and development initiaitives. Importantly, many of the strains represented in the CABI collection are also being securely held in collections of project partners in Europe and Africa, particularly those at CIRAD and UCL.

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

Molecular variability

Single-enzyme, agarose gel-based amplified fragment length polymorphism (AFLP) analysis, PCR analysis of anchored inter-simple-sequence-repeat-anchored (ISSR) DNA sequences, amplification of A+T-rich DNA (i.e. presumptive mtDNA RFLPs) and enzymatic digestion of the IGS region, while revealing limited genetic variation among the *G. xylarioides* strains, identified two major and genetically distinct groups within the CWD pathogen (see Figures 1-3 for examples of DNA profiles obtained by ISSR and IGS analysis, and dendrogram depicting the extent of genetic relationship between strains). The first comprised those *G. xylarioides* strains isolated from *C. arabica* affected by CWD in Ethiopia, the second *G. xylarioides* strains isolated from *C. canephora* affected in Uganda, DRC and Tanzania (the latter group includes one strain from *C. excelsa* in Uganda). All strains were obtained from plants affected by CWD since re-emergence of the disease on *C. canephora*. Irrespective of approach employed, no genetic variability was observed among strains of either of the two groups.

As shown in the UPGMA dendrogram (Figure 3), the four strains obtained during the earlier outbreaks in Central and Western Africa ('historical strains') were genetically distinct to those of the larger *C. arabica* and *C. canephora* groups. Of these, two from IC and Guinea appear to be genetically identical and, based on mitochondrial DNA analysis, are more closely related to the third historical strain (ATCC 15664, geographic origin unknown) than the fourth (DSMZ 62457, from CAR). The former three strains are also genetic more similar to the *C. canephora* group than to DSMZ 62457. Of note, strain DSMZ 62457 has been shown to be pathogenic to a broad range of *Coffea* species than other strains assessed and, unusually among the strains tested in plant inoculation studies by project partners, induces CWD symptoms on *C. arabica* and *C. canephora.*

All approaches applied revealed molecular profiles among the other *Fusarium* species to be clearly distinct to the *G. xylarioides* strains also to one another. Although the extent of genetic variability depended on the approach employed, particularly with regard to those strains obtained during the earlier CWD outbreaks, overall the findings correlate closely with those of genetic and biological studies, including microsatellite-based PCR analysis and sexual mating tests, undertaken by the project partners at UCL, CIRAD and in Africa (see seperate report entitled 'Synthesis of activities and findings of research activities undertaken by partners on pathgen variability and contributing to Work Package 1', other sections of the Final Report, partner reports and publications cited).

Vegetative compatibility group testing

Mutant types required for strain pairing were successfully generated for 14 of 16 *G. xylarioides* strains evaluated, providing a total of 91 separate mutant lines. The majority of these were identified as nit M types and not nit 1 and nit 3 types also required for pairing. Only one strain, IMI 375916 (ex. *Coffea arabica*, Ethiopia) produced suitable nit1 and nit3 mutants, while some lines were wild type revertants. When the nit 1 and nit 3 mutants of IMI 375916 were paired against nit M mutants of each other strain, vegetative compatibility was only observed (as would be expected) in the self pairing test between the IMI 375916 nit 1 and nit 3. Repeat attempts to generate more mutants of the required type through adaption of the mutant inducing conditions proved unsuccessful. Given the time-consuming nature of VCG testing, this particular approach was not pursued further but, where time and resources allow a more on-depth investigation, remains an option for assessing pathogen variability in future studies.

3.3 Establishing and maintaining an electronic database of baseline data relating to strains acquired

A comprehensive database of information on the fungal strains obtained during the project by the project partners and held in secure collections (namely CABI, UCL and CIRAD) was established at CABI E-UK. The database, based on *Excel*, provides information for all strains including: morphological identity of strains (to genus and if possible species level); known accession numbers, as assigned to existing strains by recognised genetic resource collections (e.g. CABI, ATCC, CBS and DSMZ) or to new strain by project partners; geographic origin (e.g. country and/or locality); host species (e.g. coffee species) or other substrate and tissue from which isolated; date of isolation and/or received by the project; and, in the case of existing strains, the donating organisation or individual. The database constitutes a valuable resource that enabled collation and secure storage of information acquired during the project and facilitated exchange of information on project findings between project partners located in Africa and Europe.

3.4 Synthesise results of research activities undertaken by project partners contributing to Work Package 1.

An overview of the activities, findings, conclusions and implications of the research activities undertaken by all project partners, including CABI, on pathogen variability as part of Work Package 1 is provided in a seperate section of the Final Report. As this encompasses the activities and findings reported here, a synthesis specific to the work of CABI is not provided here.

4. Problems Encountered

No major problems were experienced during the course of the research.

5. Technology Implementation Plan

Where possible, and as detailed in Section 6 and Appendix 1, the new knowledge, resources and methodologies developed and applied during the course of the research undertaken at CABI have been published and otherwise promoted and CABI, in association with its partners, will endeavour to continue to do so in the future. While the project outputs also have potential for uptake into future research and development initiatives, there are currently no plans to develop or release marketable products as a result of the work undertaken.

6. Publications and Papers

Publications

RUTHERFORD, M. (2006). Current knowledge of coffee wilt disease, a major constraint to coffee production in Africa. *Phytopathology* **96**, 663-666.

Poster presentations (conferences, workshops and other meetings)

BIEYSSE, D., LEPOINT, P., HAKIZA, G., KALONJI, A., TSHILENGE, P., MUNAUT, P., JANZAC, P. and ROUSSEL1, V. (2006). Coffee Wilt Disease: a major constraint to coffee production in Africa. Presented at EFS9 Symposium, Wageningen, 19-22 Sept. 2006.
RUTHERFORD, M., J. CROZIER, A. BUDDIE, J. INESON, S. LEA and J. FLOOD. (2005). Coffee Wilt Disease. Presented at the Regional Coffee Wilt Stakeholders Workshop, Nairobi, Kenya. 8–9 December 2005.
FLOOD, J. (2003) Fusarium wilts of tropical perennial crops- an overview of management strategies: Challenges to the management of *Fusarium* wilts of tropical crops. International Fusarium Wilt Symposium, Salvador, Brazil Sept. 2003 (presented by J. Flood, CABI E-UK).
FLOOD, J., RUTHERFORD, M., BUDDIE, A., BRAYFORD, D., INESON, J., CANNON, P., HAKIZA, G., BIEYSSE, D. and KALONJI-MBUYI, A. (2003). *Fusarium* wilt of coffee in Africa - re-emergence of an old problem. International Fusarium Symposium, Sydney, Australia, February 2003 (presented by J. Flood, CABI E-UK).

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 (presented by M. A. Rutherford, CABI E-UK).
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, CABI E-UK).
RUTHERFORD, M.A., BUDDIE, A., INESON, J., CROZIER, J. and FLOOD, J. (2004). Regional Coffee Wilt Programme: CABI UK Centre research activities: progress. Regional Coffee Wilt Programme Stakeholders' and Planning Workshop, Nairobi, Kenya, Nairobi, 9-13 November 2004 (presented by M. A. Rutherford, CABI E-UK).
RUTHERFORD, INESON, J., BUDDIE, A. and FLOOD, J. (2003). Regional Coffee Wilt Programme: CABI UK Centre research activities. Regional Coffee Wilt Programme Stakeholders' and Planning Workshop, Nairobi, Kenya, 3-5 December 2003 (presented by M. A. Rutherford, CABI E-UK)
RUTHERFORD, M.A., INESON, J., BUDDIE, A. and FLOOD, J. (2002). Regional Coffee Wilt Programme: CABI UK Centre research activities. Regional Coffee Wilt Programme Stakeholders' and Planning Workshop, Nairobi, Kenya, 13-14 November 2002 (presented by M. A. Rutherford, CABI E-UK).
GIRMA, A.S., FLOOD, J., HINDORF, H., BIEYSSE, D., SIMONS, S. and RUTHERFORD, M. (2006). Tracheomycosis (*Gibberella xylarioides*) - a menace to world coffee production: evidenced by cross inoculation of historic and current strains of the pathogen. *Proceedings of the 21st International Scientific Conference on Coffee Science* (ASIC), 11–15 September 2006, Montpellier, France. pp 1268-1276
KILAMBO, D. L., NG'HOMA, N. M., MTENGA, D. J., TERI, J. M., NZALLAWAHE, T., RUTHERFORD, M. and MASUMBUKO, L. (2006). Progress towards searching for durable resistance to Fusarium Wilt (*Fusarium xylarioides*) in *Coffea canephora* germplasm in Tanzania. *Proceedings of the 21st International Scientific Conference on Coffee Science* (ASIC), 11–15 September 2006, Montpellier, France. pp 1386-1389.
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.A. (2006) Epidemiology and variability of the coffee wilt pathogen. pp 42-44 in: Sweetmore,A., Kimmins, F. and Silverside, P. (eds). *Perspectives on Pests II: Achievements of research under UK Department for International Development Crop Protection Programme 2000–05.* Natural Resources International Limited, Aylesford, UK. 260+xvi pages. ISBN: 0-9546452-7-8
RUTHERFORD, M., FLOOD, J., LEA, S. and CROZIER, J. (2005). Coffee Wilt Disease. Advisory leaflet. 10,000 copies. 8 pp. Egham, CAB International.
UNDERWOOD, F. (2003). 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.

7. Conclusion

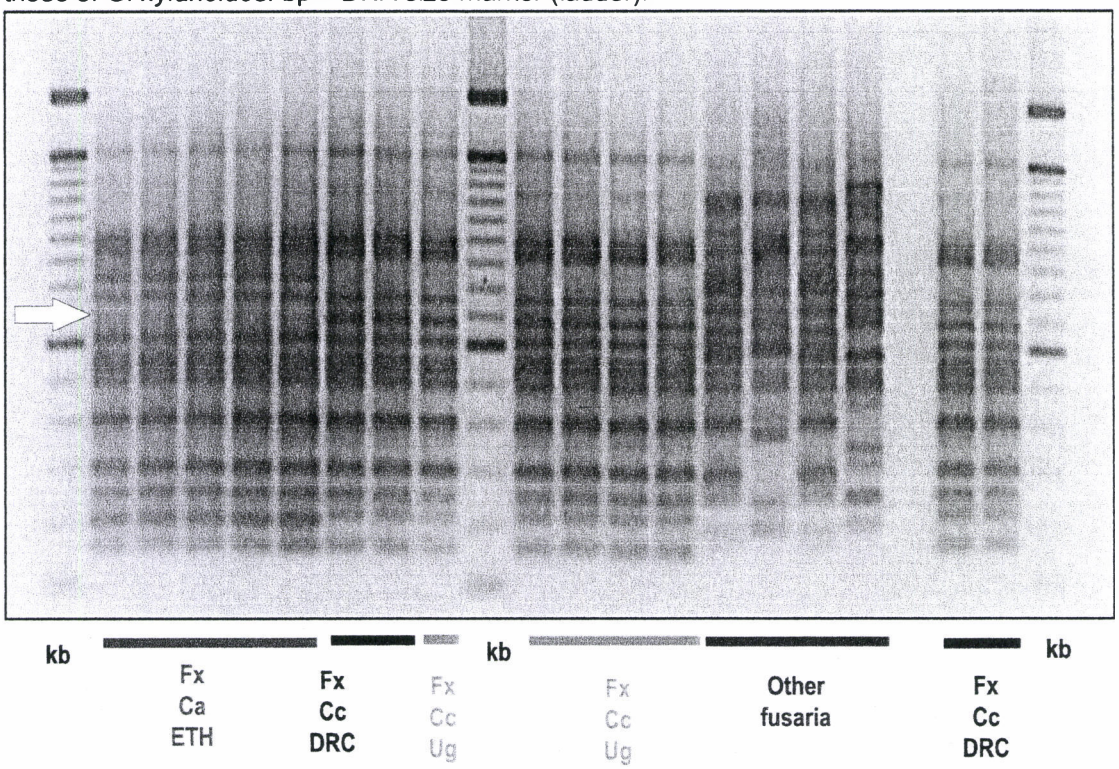
Activities undertaken by CABI as a partner during this project have led to a number of key outputs being produced, in terms of generation of new knowledge of the coffee wilt pathogen and tools and resources invaluable to the research completed by CABI and its partners. Specifically, they have led to the establishment and secure storage of a comprehensive collection of purified fusaria obtained from a range of coffee species across many countries of East, Central and West Africa, namely those currently and previously affected by CWD.

Many of these strains have been characterised from a morphological and genetical perspective by mycologists, plant pathogists and other scientific experts from CABI, CIRAD, UCL, CORI, UCL and other organisations. A comprehensive database (based on *Excel*) has been established to enable ready access to key information regarding these strains, not only in relation to the origin and identity of strains but also their attributes as previously known and, importantly, as revealed by the various scientific approaches employed during the project. Possibilities exist for further developing this information resource as and when new research initiatives are undertaken and new knowledge is acquired.

The various molecular approaches employed by CABI were consistent in clearly discriminating the various *Fusarium* species recovered form coffee. They also confirmed the suggestions of preliminary work undertaken at CABI in the late 1990s, in that two genetically distinct populations of *G. xylarioides* are associated with current CWD outbreaks in East and Central Africa. However, and in parallel with genetic and other studies undertaken at CIRAD and UCL, they did not reveal genetic diversity within either of these populations. Furthermore, the close relationship between the two populations and coffee species indicates a level of host specialisation that, in terms of current efforts to develop host resistance to the disease, is a positive finding. The existence of host specificity is also supported by pathogenicity studies undertaken to date and involving assessment of a range of the project strains, where the majority exhibit pathogenicity only to the host coffee species from which they were isolated in the field. The findings of research undertaken at UCL to investigate the role of the sexual cycle of the pathogen are also very relevant and have implications with regard to disease magagement in that, while the fungus has been confirmed as heterothallic and the sexual cycle (as denoted by the production of perithecia) can function *in vitro*, sexual incompatibility exists and correlates with the genetic variability observed to date.

The outputs generated by the project activities have already contributed to, and accelerated, the search for a solution to CWD as a devastating constraint to current coffee production. They also provide a sound basis on which future research and development efforts to tackle this and related diseases may be built, specifically with regard to our increased capacity to identify and monitor the differing forms of the pathogen and to identify, develop and deploy coffee with suitable host resistance. It will therefore be of immense importance in supporting and sttrenghtening the breeding programmes already active in affected regions of East and Central Africa.

Figure 1. DNA profiles obtained for fusaria isolated from coffee following amplification of total DNA with inter simple sequence repeat (ISSR) primer CCA. While *F. xylarioides* (the presumed anamorph of *G. xylarioides)* strains, denoted by 'Fx' are generally very similar, those from *C. arabica* ('Ca') in Ethiopia ('ETH') show a slightly different DNA profile (e.g. at points denoted by arrows) to that for strains from *C. canephora* ('Cc') in Uganda, DRC and Tanzania (the former tow denoted by 'UG', and 'DRC' respectively on the gel image). Other fusaria, including *F. stilboides, F. lateritium* and *F. oxysporum*, have very different profiles to those of *G. xylarioides*. bp = DNA size marker (ladder).



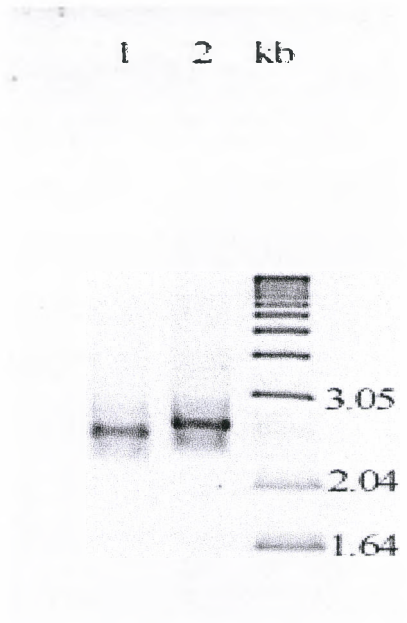


Figure 2. DNA banding patterns produced by PCR amplification of the ribosmal DNA (rDNA) intergenic spacer (IGS) region of *G. xylarioides* strains from coffee. Strains originating from *C. arabica* in Ethiopia (lane 1) show a smaller IGS fragment than those obtained from *C. canephora* in Uganda, DRC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

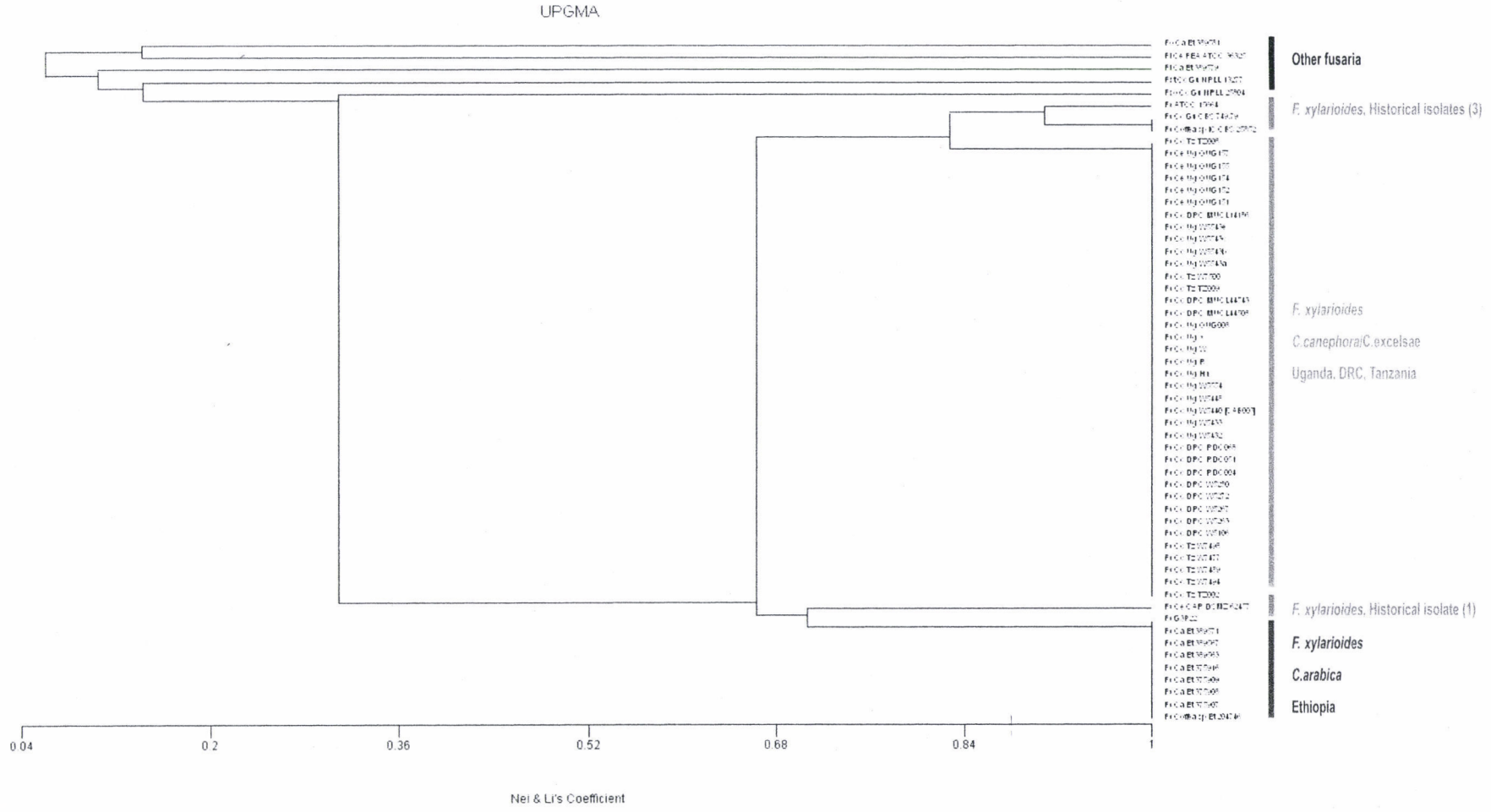


Figure 3. UPGMA dendrogram showing % genetic similarity between fusarium strains obtained from coffee affected by CWD. The dendrogram was based on mitochondrial DNA RFLP data and derived using Nei and Li's genetic distance coefficient *(Abbreviations: Fx, F. xylarioides* (presumed anamorph of *G. xylarioides); Fo, F.oysporum; Fs, F. solani; Fst, F. stilboides; Fl, F. lateritium; Ca, C. arabica; Cc, C. canephora; Ce, C. excelsa Et, Ethiopia; Ug, Uganda; DRC, Democratic Republic of Congo; Tz, Tanzania, Gu, Guinea; CAR, Central African Republic; IC, Ivory Coast; FEA, French East Africa)*