# 5
# Establishing a CWD Collection

*Order and simplification are the first steps toward the mastery of a subject.*[1]

## 5.1 Main Findings

- A collection of more than 300 purified (monosporic) anamorphic (asexual form) and teleomorphic (sexual form) strains of coffee wilt disease (CWD) was assembled throughout the research programme.
- The collection will serve as a reference library for future studies.
- A small number of strains, originally obtained from *Coffea canephora* affected by CWD in the Democratic Republic of Congo (DRC), Central African Republic (CAR), Guinea and Côte d'Ivoire 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 CAB International UK.
- A number of other Fusarium species are pathogenic to coffee: *F. oxysporum, F. solani, F. stilboides* (coffee bark disease), *F. lateritium* and *F. decemcellulare*. All of these species were recovered from CWD-affected trees.
- Several strains received at CAB International as *F. xylarioides* were found to belong to other Fusaria (namely *F. solani*, *F. stilboides* and *F. lateritium*) or to have been mixed cultures.
- Accompanying the disease collection, a comprehensive database of information on the fungal strains obtained during the project by the project partners was established at CAB International UK in 2002 and is now shared by CAB International, Université Catholique du Louvain (UCL) and Centre de Coopération Internationale en Recherche Agronomique pour le Développement (CIRAD).

## 5.2 Collecting CWD

For the sake of long-term research continuity, it is very important to bring together and maintain a reference collection of CWD strains from different locations as well as other related coffee diseases. Ideally, such collections should have been made routinely over the decades so that variations in the disease could be tracked. Fortunately, however, a few samples had been retained from the original outbreaks in the first half of the 20th century. Without those we would have no idea if the new disease was related to the historical outbreaks.

In the field, samples were routinely taken immediately below the bark from pieces of coffee wood exhibiting blue-black discoloration, which is a diagnostic feature of infec-

[1] Thomas Mann, *The Magic Mountain*.

tion by CWD. Where possible, isolations were made in country, with an original, purified culture being retained for in-country activities and a subculture being sent to CAB International UK for confirmation of identity to species level and for use in research activities undertaken at CAB International.

The collections were made of samples made by project scientists together with those donated from project partners in France, Belgium, Uganda and DRC, as well as other research organizations and recognized culture collections around the world (including stocks already held at CAB International).

Fungal isolations were mostly made from coffee plant material by removal and rehydration (if the samples are exceptionally dry, as in the case of wood) of root, shoot or wood pieces in sterile distilled water for 3–5 min. These were then surface sterilized by immersion in sodium hypochlorite solution (1.4% a.i.) for 1–2 min, rinsed several times in sterile distilled water and one to four small pieces of material placed on tap water agar (TWA) medium (Booth, 1971). Agar plates were incubated at 25°C (under daylight fluorescent light tubes) for several days and emerging fungal colonies aseptically transferred to synthetic low-nutrient agar (SNA; Nirenberg, 1976) medium and cultured at 25°C.

Cultures were single-spored by removing fungal material from an SNA culture in a loopful of sterile distilled water and streaking the loop across a TWA plate. Plates were incubated at 25°C for 24–36 h and, with the aid of a binocular microscope, a single spore with emergent germ tube removed on an agar block (using a scalpel) and transferred to fresh SNA medium. The block was examined under a microscope at 40× magnification to ensure that a single spore was then removed and the SNA plates had been incubated at 25°C. For the purposes of identification of Fusaria to species level, purified isolates were grown at 25°C on SNA and potato sucrose agar (PSA, Booth, 1971) medium; Fusaria usually producing pigmentation characteristic of particular species on the latter. For isolation of Fusaria from soil, a few grams of air-dried soil were sprinkled on to TWA or SNA plates which were then incubated at 25°C. Fungal colonies emerging from soil particles were subcultured on to fresh SNA medium for purification and/or identification. Where bacterial contamination was considered problematic, TWA medium was amended with either 150 mg/l streptomycin sulfate (when isolating from stem pieces, roots and twigs) or 300 mg/l (when isolating from soil) to inhibit bacterial growth.

Where possible, the identity of purified strains was determined to species level on SNA and PSA based on established morphological characters.

In this way, a collection of more than 300 purified (monosporic) anamorphic (asexual form) and teleomorphic (sexual form) strains of CWD was assembled throughout the research programme. Of all the isolates obtained, 284 are currently held at CAB International UK. The majority (249) of these were isolated from stem wood (Table 5.1). Many were successfully identified to species level by CAB International UK scientists or by project partners, or are suspected to be a particular species based on information received on receipt (e.g. accession data provided by other collections).

A small number of strains, originally obtained from *C. canephora* affected by CWD in DRC, CAR, Guinea and Côte d'Ivoire 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 CAB International UK.

**Table 5.1:** Isolates obtained from coffee trees exhibiting coffee wilt disease (CWD) symptoms.

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

A total of 206 CWD isolates were thus consolidated from *C. canephora* trees cultivated in Tanzania, Uganda, DRC and Guinea, 21 from *C. arabica* trees in Ethiopia and 10 from C. *excelsa* trees in Uganda and the CAR. In addition, isolates were obtained from wood and root samples collected from coffee trees with and without external CWD symptoms at a farm in Mayuge, Uganda. This farm was one of six on-farm sites, where the development of CWD has been monitored spatially and temporally since 2002. Although both Arabica and Robusta are cultivated in Tanzania, CWD was only observed on the latter in Muleba, Bukoba and Karagwe. Conversely, although Arabica is cultivated much more widely than Robusta in Ethiopia, only Arabica is affected by CWD in that country.

A total of 60 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 representative subset for in-depth study of variability using a number of approaches as described elsewhere in this report. Seventy strains, including members of the representative group, were deposited for long-term storage under liquid nitrogen and in a freeze-dried state in the Genetic Resources Collection at CAB International UK.

The culture collection, thus constructed, provided a valuable reference resource for the project and will be maintained for the benefit of future research and development initiatives. Importantly, many of the strains represented in the CAB International collection are also being securely held in collections of project partners in Europe and Africa, particularly those at CIRAD and UCL.

## 5.3 Other Bark Diseases Related to CWD

A number of other Fusarium species occur on coffee, including *F. oxysporum*, *F. solani*, *F. stilboides* (coffee bark disease), *F. lateritium* and *F. decemcellulare*. All are pathogenic

to coffee and all have been recovered from CWD-affected trees. Of note, several strains received at CAB International as the anamorph (asexual form) of *G. xylarioides* were found, through morphological examination, to belong to other Fusaria (namely *F. solani*, *F. stilboides* and *F. lateritium*) or to have been mixed cultures.

How these fungi interact with CWD – if there is symbiosis, antagonism or simply coexistence – is unclear. This complexity can lead to confusion in diagnosis, which requires considerable expertise. Field diagnosis is not enough, samples need to be taken and grown in an appropriate agar medium, where their colour and form permit the different species to be distinguished.

A total of 16 isolates belonging to other Fusaria were obtained from coffee trees in CWD-affected areas (Table 5.2). Of these, six (*F. stilboides*, *F. lateritium*, *F. oxysporum* and *F. decemcellulare*) were obtained in Ethiopia and two (*F. solani*) in Tanzania. A number of these isolates were recovered from coffee trees exhibiting external (and in some cases internal) symptoms similar to those of CWD and, in some cases, from wood pieces from which *F. xylarioides* was also recovered. This suggests that they may coexist on the crop as components of a disease 'complex'. However, the precise role of each of these species, all of which may individually cause specific diseases of coffee, remains unclear. They may collectively contribute to the development or expression of CWD-like symptoms, which are often not definitive with respect to disease diagnosis. Perhaps, some species reduce host vigour or provide entry points for *F. xylarioides*.

Alternatively, they may invade host tissues as opportunistic pathogens or saprophytes subsequent to infection by *F. xylarioides*. As far as is known, the pathogenicity of these isolates to *C. canephora*, *C. arabica* and *C. excelsa* has not yet been tested, either alone or in combination with pathogenic forms of *F. xylarioides*. Nevertheless, based on these isolations and feedbacks from in-country counterparts, it is strongly suspected that symptoms caused by other Fusaria are sometimes mistakenly attributed to CWD. This highlights the need, first, to increase awareness and understanding of the various Fusaria that may affect coffee and enhance capacity among in-country counterparts to recognize and differentiate the various symptoms that may be encountered. Second,

**Table 5.2:** Fusaria other than *Fusaria xylarioides* obtained from coffee trees exhibiting coffee wilt disease (CWD) symptoms.

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

the in-country disease surveys should be supported by isolation of fungal organisms from diseased plants and any fungi identified to species level as a means of ascertaining what may be responsible for the symptoms observed.

## 5.4 The Problem of Isolation from the Soil

The various Fusaria, including *F. xylarioides*, were routinely isolated with relative ease from coffee wood pieces excised from symptomatic trees. In contrast, *F. xylarioides* could not be isolated from soil particles sprinkled on to agar media, including a medium selective for Fusarium species (Komada, 1975). Plates usually became overgrown by other Fusaria without CWD becoming apparent, a recurring problem where pot- and field-based experimentation were concerned (see Chapter 10). These Fusaria may actively compete with or inhibit the growth of *F. xylarioides* and therefore restrict or inhibit its growth or simply obscure its presence. This is supported by reports that *F. xylarioides* was readily isolated from soil that had been collected from a field site affected by CWD, steam sterilized and subsequently inoculated with the pathogen. However, the fungus could not be re-isolated from soil that had not been sterilized prior to inoculation (Hakiza, personal communication). The problem could not, as had been hoped, be resolved by serial dilution plating, despite several attempts being made.

In many such cases, it was suspected or known that the pathogen was in fact present in soil, as shown by the development of CWD symptoms on susceptible coffee seedlings transplanted into the soil. While the use of susceptible plants as a means of 'baiting' *F. xylarioides* from soil is usually effective, a more rapid and straightforward means of confirming the presence of, and isolating, the fungus from soil is necessary to support fundamental and critical studies on, for example, the ability of *F. xylarioides* to remain viable in soil and persist as a source of inoculum.

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

## 5.5 A Comprehensive CWD Database

Accompanying the disease collection, a comprehensive database of information on the fungal strains obtained during the project by the project partners was established at CAB International UK in 2002 and is now shared by CAB International, UCL and CIRAD.

Data input to the database is related to: mycological identity; geographic origin (e.g. country, locality); host species (e.g. coffee species) or other substrate and tissue from which it is isolated; date isolated and date received at CAB International UK; details of donor and known accession numbers, including those relating to the internationally

recognized collections at CAB International, the American Type Culture Collection (ATCC), the Centraalbureau voor Schimmelcultures (CBS) and Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ – the German Collection of Microorganisms and Cell Cultures) numbers. Background notes referring to, for example, unusual mycological attributes of an isolate were also input where considered appropriate.

The database thus 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.

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

## 5.6 Conclusions

A comprehensive collection of CWD and other Fusarium disease strains was collected together and preserved to serve as a long-term reference for current and future studies on this disease complex. Principal strains are maintained at CAB International (UK), CIRAD (France) and UCL (Belgium), and anyone interested in receiving isolates for research should apply to one of these institutes.

Globally, such collections are under threat because of shortage of funds. It is clear, however, that when a new disease breaks out, they provide extremely useful information. Ideally, in the future, these collections should not only be maintained but augmented through regular collecting trips to monitor the progress of the disease, and with the powerful molecular techniques now available, their origins and spread could be plotted and predicted.