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Full Length Research Paper

Determination of cultural and biometrical characters of *Fusarium* species isolated from plant material harvested from coffee (*coffea canephora* Pierre.) infected with CWD in Democratic Republic of Congo

L. Tshilenge[1]*, A. Kalonji[1,2] and P. Tshilenge[2]

[1]Department of Genetic and Plant Breeding, General Commissariat for Atomic Energy, P. O. 868, Kinshasa XI, Democratic Republic of Congo.
[2]Faculty of Agriculture, Unit of Phytopathology, University of Kinshasa, P. O. 117, Kinshasa XI, Democratic Republic of Congo.

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The Coffee Wilt Disease (CWD) is vascular disease due to *Gibberella (Fusarium) xylarioides* has decimated coffee plantations in the Democratic Republic of Congo. The authors determined the characters of different species of *Fusarium* isolated from Robusta coffee trees affected by this disease, to establish their causality. The cultural and biometricals characters have been studied. Differences were observed between the stock of *F. xylarioides* and those of *Fusarium lateritium, Fusarium solani, F. falciforme,* and, *Fusarium stilboides*.

Key words: CWD, Robusta coffee, *Fusarium xylarioides*, *Fusarium lateritium, Fusarium solani*, *Fusarium falciforme, Fusarium stilboides*.

INTRODUCTION

The large coffee plantations producing areas in the provinces of Equateur, North Kivu and Orientale Province in Democratic Republic of Congo (DRC), are heavily attacked by the CWD, a disease that causes the decline of coffee the Robusta variety. This disease, caused by a fungus, *Gibberella xylarioides* Heim and Saccas, anamorph *Fusarium xylarioides* Steyaert, has been described by various authors since its discovery in DRC (Steyaert, 1948; Fraselle, 1950) in West and Central Africa (Saccas, 1951) until recently to mark its resurgence in the DRC (Katenga, 1989 Tshilenge et al., 1998).

Since its discovery in 1939 in Aba, a town on the border with Sudan, CWD has been combated and controlled from the time it was reported in the coffee plantations of the National Institute for Agronomic Study in Congo (INEAC) in Yangambi in 1949 (Fraselle, 1950). Control strategies that have allowed this eradication were essentially preventive and mechanical order. They consisted of pulling, cutting and burning, in situ, subjects patients upon detection. After several decades of successful application of this struggle, we saw the resurgence of CWD in the district of Haut-Uele circa 1982 (Katenga, 1987). From this new hearth, it has spread to almost all coffee plantations in the north and north-eastern DRC, with average rates of attack has changed from 19.3% to 100% depending, on the areas Culture (Tshilenge et,al., 1998).

Several hypotheses are made about the causes of this resurgence. Among them was thought possible role in the pathogenesis of other species of *Fusarium* (Maraite, 2003; and Tshilenge et al., 2004) frequently isolated side of *F. xylarioides* in Robusta coffee (Girma, 2004; Rutherford, 2004; Tshilenge et al., 2004; Serani et al. 2007).

The variability of symptoms that point above the final stage of decay of coffee and the presence of various *Fusarium* spp. beside *F. xylarioides* during isolations in

*Corresponding author. E-mail: lucktshilenge@yahoo.fr.Tel: +243 81 9480310.

Table 1. List of *Fusarium* spp. studied.

Number	Species	Strain	Origin in DRC	Date of harvest by [1]	Date of isolation by [2]	Identified by [3]
01	*F. lateritium*	-	Isiro	17/12/02 K.T.D.	16/01/03 T.	T. and FM.
02	*F. falciforme*	MUCL43880	Bas-Congo	12/03/02 K.T.D.	17/03/02 HM.	HM. and FM.
03	*F. solani*	-	Isiro	17/12/02 K.T. D.	16/01/03 T.	T. and FM.
04	*F. stilboides*	SR17/09	Butembo Njiya	12/09/02 NV.	17/12/02 L.	L.
05	*F. xylarioides*	B10101(2)J	Beni-Mutwanga	02/12/02 K.T. D.	02/01/03 T.	T.

[1] K = Kalonji; T = Tshilenge; D = Dibue; P = Pochet; NV = Ndungo Vigheri. [2] L = Lepoint; T =Tshilenge. [3] L = Lepoint;; T = Tshilenge.

the stems of affected coffee trees, suggests the role of these different species in this condition. This could contribute to a reorganization of management control by including this *Fusarium* spp. in the screening program of plant material for durable, resistance. This work aims to identify principal cultural characters and biometrical Fusarium species present in the coffee wilt disease, namely: *F. lateritium, F. solani, F. falciforme*, *F. stilboides* and *F. xylarioides*.

MATERIALS AND METHODS

Fusarium, spp., studied

Isolates of *Fusarium* spp. were made from pieces of wood Robusta coffee suffering from CWD collected during surveys conducted from 2002 - 2003 in the coffee growing regions of the DRC. We used samples shaped slices of varying dimension (3 - 5 cm long and 2 - 4.5 cm in diameter), depending on the dimension of the stems. These were surface-disinfected with 70% ethanol and then flamed quickly to evaporate excess alcohol, then split in two, under aseptic conditions using a carpenter's bevel and a rubber mallet, trying to go through areas with "black ribbons" subcortical bare symptoms of CWD in coffee. Inside the wood, color dark places, tiny fragments of a few millimeters were taken using a scalpel tip n° 11 and placed on water agar medium Streptomycin (Merck Agar ®: 15 g; H_20: 1000 ml Streptomycin: 100 mg). After issue of mycelium, the ends of hyphae were subcultured on medium Synthetic Nutrient Agar (SNA KH_2PO_4: 1 g; KNO_3 1 g; $MgSO_4.7H_2O$: 0.5 g, KCl: 0.5 g; Glucose: 0, 2 g, Sucrose, 0.2; Agar Merck ®, 20 g, H20 1000 ml). A collection consists of different inbred strains is maintained on this medium under paraffin tubes. Table 1 lists the sample studied.

Macroscopic characterization

The culture medium containing potato is conducive to the expression of macroscopic characters (Maraite, 2003; Tshilenge et al., 2004). The composition of the medium used, based on the formula for Lacy et al. Cited by Tuite (1969) is as follows: potato powder dehydrated: 20 g; Dextrose: 20 g, Agar 20 g, H_20 1000 ml. The pH before sterilization was 5. The procedure was as follows: The peeled potato was washed with tap water, sliced and then rinsed with distilled water. Slices are dried in an oven at 65°C until constant weight. Then they were crushed in the mill brand "Thomas Scientific (USA)"and" grinder" (Fritsch, Germany). The medium was then sterilized in an autoclave at 120°C during 20 min. After sterilization it was distributed under a laminar flow near a Bunsen burner flame, in Petri dishes 9 cm in diameter. After cooling, the boxes were returned to avoid condensation on, the lids. Sub-cultures for the study of macroscopic characters were obtained from transplanting a slice of 5 mm in diameter cut with a cork borer at the periphery of the mycelium of the cultures of stock-girls previously obtained on SNA. The slices were placed in the center of the Petri dish containing agar medium potato described above. The observations were registered daily in daylight. They focused on radial growth, the contour of the disc and mycelial pigmentation of the mycelium.

The diameter mycelial disc was measured every day until day 10 which corresponds to the total occupation of the Petri dish. This was done to calculate the daily rate (cm /day). The recorded data were analyzed using the software R according to the ANOVA model with a factor. The comparison of means was performed by LSD test. Cultures were incubated at 27 ± 3°C in the dark for 10 days. The description of the color of aerial mycelium and the reverse of the colony was made on the 10th day of culture using the scale proposed by Nelson et al. (1983). The outline of the colonies was described relative to the diagram presented by Ainsworth and Bisby's (1971).

Microscopic, characterization

The analysis of microscopic characters were made from subcultures incubated at room temperature (± 27°C) for 7 days on medium synthetic nutrient agar (SNA). From the strains obtained after resuscitation daughter of stem-mothers, we conducted the production of subcultures by lining out grooves using a platinum loop on SNA. Transplanting in scratches can be rubbed a large area and detect possible contamination. The Petri dishes were returned, labeled and tightly closed with the parafilm and then incubated at ambient laboratory temperature (± 27°C). The biometrical characteristics of conidia were observed on microscopic sections made from fragments taken from subcultures in places where prior observation at the back of the box revealed a high density of conidia.

The sections were mounted on a glass slide in a drop of lactophenol blue and observed under a microscope (Olympus BX 40) equipped with a camera (Olympus OM-4) with 10x eyepiece fitted with a scale micrometer and the 40x. The measures, taken on a sample of 40 conidia, brought to the macroconidia, the frequency of different shapes, length and width at the widest section and the number of partitions. The shape, length and width of microconidia were also described and their frequency. These have been described with reference to the morphological description given by Ainsworth and Bisby's, (1971).

In order to study the density of conidia produced by each species was taken in the preparation of suspensions of conidia from subcultures kept in incubation for 7 days. Sporulation of the fungus was evaluated using the direct method of counting with the hemacytometer or Thoma cell.

Table 2. Observations on cultures of different species.

Espèce	Stock	Color		Contour
		Of aerial mycelium	Of the underside of culture	
F. lateritium	-	White	cream	toothed, laciniate
F. falciforme	MUCL43880	White	orange	slightly sinuous
F. solani	-	cream	carmine red	sinuous
F. stilboides	SR17/09	purplish	purplish	sinuous
F. xylarioides	B10101(2)J	White	beige blue center	slightly sinuous

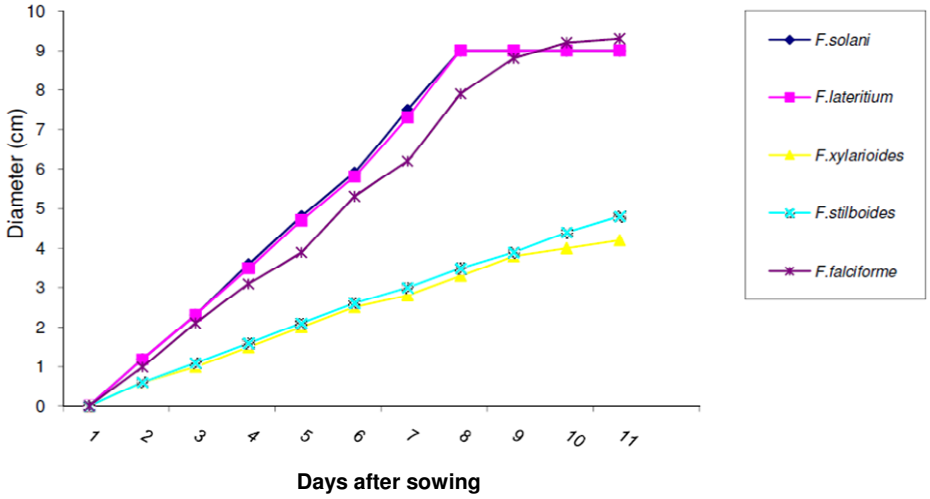

Figure 1. Growth rate on PDA of different *Fusarium* spp. associated with coffee wilt disease in DRC.

RESULTS

Macroscopic characters

Mycelial, pigmentation and contour

Observations on cultures of different species have revealed variability in cultural characteristics observed (Table 2). Examination of the characters presented by stocks of *Fusarium* spp. reveals that the point of view of the outline of the colonies, it is slightly sinuous for *F. falciforme* and *F. xylarioides*, and more sinuous and the *F. solani* and *F. stilboides*. The colonies of *F. lateritium* are characterized by an irregular and deeply indented at laciniate.

In terms of pigmentation, the color of aerial mycelium highlights the *F. lateritium* and *F. stilboides* by their red color and purple respectively. In contrast, other species have a white mycelium. It is more for the color of the underside of culture, a clear difference between species. *F. xylarioides*, pigmentation beige, blue in the center of the underside of culture is distinguished from other species with a single color each setback. This is the case with a reverse *F. solani* carmine red, orange *F. falciforme* presents a setback while *F. lateritium* and *F. stilboides* have setbacks and cream and purple, respectively.

Radial,growth

Examination of the radial growth rate (Figures 1 and 6), calculated on the linear phase of the evolution of the growth curve, shows the differences between speciesthat appear after the 2nd date. Some species have maintained a steady trend in their growth and two different groups each day have been observed. Analysis of variance of the growth rate shows a clear difference ($LSD_{0,01}$ = 0,1) between the group *F. solani, F. falciforme* and *F. lateritium* one hand and that of *F. xylarioides* and *F . stilboides* other. The first is characterized by faster growth (0.96 - 1.11 cm/d) than the second (0.63 cm/d). Within the first group, the *F. falciforme* is slower.

Microscopic characters

Frequency of forms conidia

The different forms of macroconidia observed are grouped into curved, sickle and Fusoid. The curved

Table 3. Macroconidia shape frequency of different *Fusarium* spp. associated with coffee wilt disease in DRC.

Species	Sock	Shape		
		Curved (%)	Sickle (%)	Fusoid (%)
F. lateritium	-	N.O.*	100	N.O.*
F. falciforme	MUCL43880	N.O.*	N.O.*	N.O.*
F. solani	-	N.O.*	N.O.*	N.O.*
F. stilboides	SR17/09	N.O.*	90,5	9,5
F. xylarioides	B10101(2)J	94,10	5,90	N.O.*

N.O.*= Not Observed.

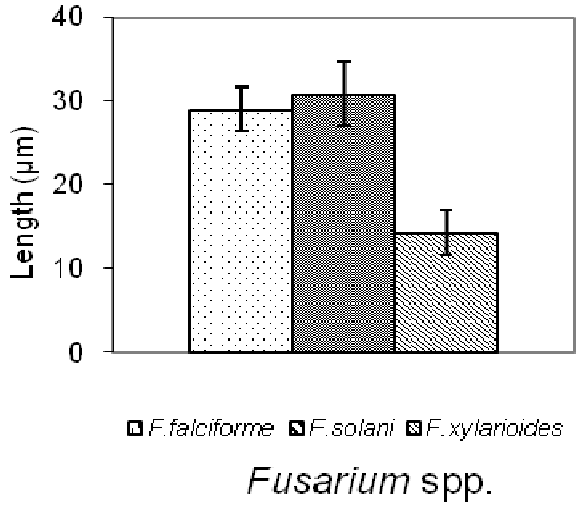

Figure 2. Lenght of macrocondia of *Fusarium* spp. associated with CWD in DRC 7 days culture on SNA.

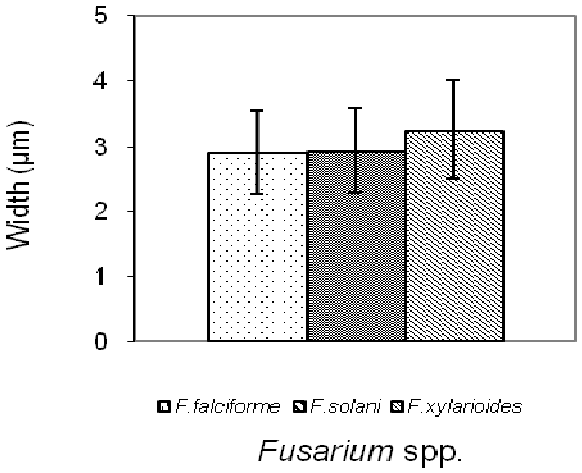

Figure 3. Lenght of macrocondia of *Fusarium* spp. associated with CWD in DRC 7 days culture on SNA.

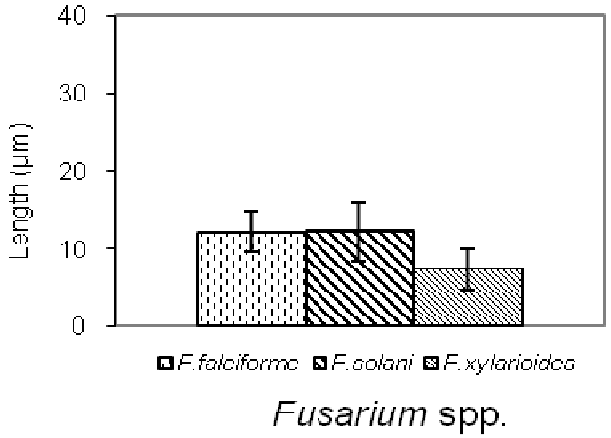

Figure 4. Lenght of macrocondia of *Fusarium* spp. associated with CWD in DRC 7 days culture on SNA

shape approximates that of a half-circle pinched while the sickle shape is that of a sickle and Fusoid resembles a spindle. Table 3 presents the observed frequencies for these different morphologies.

An examination of the morphology of macroconidia and frequency (Table 3) shows some clustering of species. The curved shapes and Fusoid are absent in the *F. lateritium,* which presents cons falcate macroconidia in 100% of cases. The *F. stilboides* falcate macroconidia produced at 91% against 9% Fusoid Macroconidia were not observed in cultures *F. solani* and *F. falciforme*. In *F. xylarioides*, it was found predominantly curved macroconidia (94%) followed by sickle (6%).

Dimension of conidia

Macroconidia

The analysis of the length and width of macroconidia on SNA shows differences between species ($LSD_{0,01}$ = 2.8). *F. stilboides* and *F. lateritium* present the macroconidia measuring 29.04 - 30.85 µm in length on one hand and *F. xylarioides* with macroconidia measuring 14.32 µm in length on average. Regarding the width of macroconidia, significant differences could not be established between

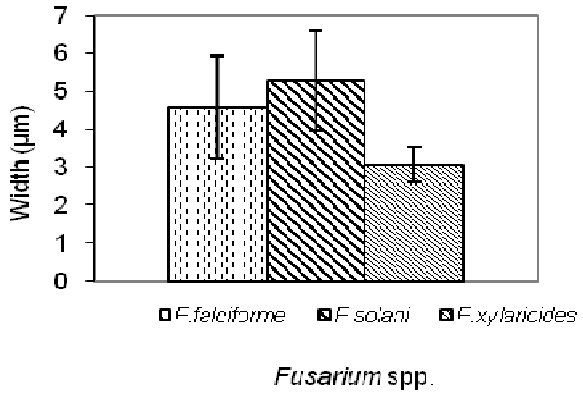

Figure 5. Width of macrocondia of *Fusarium* spp. associated with CWD in DRC 7 days culture on SNA

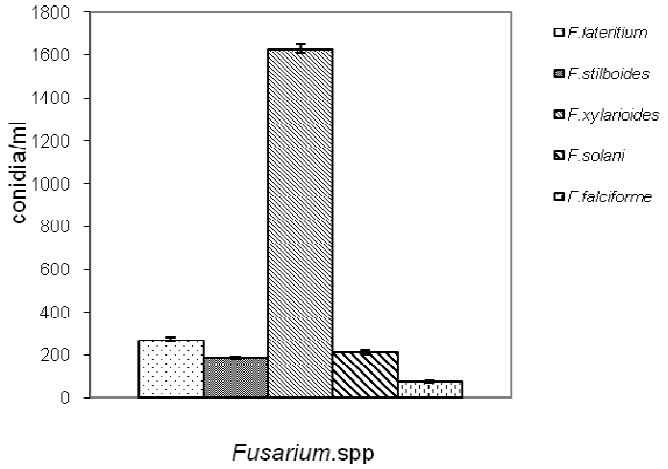

Figure 6. Production of conidia of Fusarium spp. associated with CWD in the DRC 7 days after culture on SNA

the different species of *Fusarium*. Similarities between *F. lateritium* and *F. stilboides* explained by the fact that these two species belong to section XII (lateritium).

Microconidia

The analysis of values measured indicates for *F. falciforme* and *F. solani* microconidia measuring 12.07 – 12.12 µm x 4.53 – 5.26 µm. Those of *F. xylarioides* showed shapes ranging from cylindrical, curved and allantois to measure length 7.27 and 3.07 µm wide. Significant differences were not observed between these values.

Density sporulation

Examining the results of Figure 1 shows a large difference between the different *Fusarium* species ($LSD_{0.01}$ = 2.5). Strong production is observed in *F. xylarioides*. Low production is recorded in the *F. falciforme*. The *F. lateritium, F. stilboides* and *F. solani* showed medium production ranging from 188 - 270 conidia. Given these results, it should be noted that the nature of the culture medium significantly affects sporulation. The SNA medium is conducive to sporulation of *F. xylarioides*. According to Marshall Ward, cited by Saccas (1951), the malleability of *Fusarium* species and their ability to adapt to other conditions are further increased.

DISCUSSION

The study of cultural characters and biometrical *Fusarium* species associated with CWD in the Democratic Republic of the Congo (DRC) has shown mixed results. From the perspective of cultural features (radial growth, pigmentation and contour of aerial mycelium) on PDA, differences between species were observed on the radial growth and pigmentation, while the contour of mycelial discs was not very different for all species. In terms of radial growth, the differences have led to a reunion between species. The first group consists of fast growing species. This is the case *F. solani*, *F. lateritium* and *F. falciforme*. The *F. xylarioides* and *F. stilboides* are characterized by slow growth. Such variability has been observed by various authors (Saccas, 1951; Booth, 1971; Bieysse, 2004; Girma, 2004; and Tshilenge et al., 2004). The characters of *Fusarium* show polymorphism and extreme variability, the extent of their changes, their special characters at different ages, their flexibility vis-à-vis all the external factors (chemical composition, concentration and reaction of substrate, temperature, humidity, light, ...) greatly complicate their morphological classification and greatly diminish the value. The population density of *Fusarium* on the plant and the soil is a function of plant resistance and the amount of initial inoculum (Leslie and Summerell, 2006).

Experiments on the pigmentation of *Fusarium* species in culture have shown that the coloration of the underside of mycelial colonies depends on the species (Leslie and Summerell, 2006). The species belonging to the same group (*F. xylarioides*, *F. stilboides*, *F. lateritium* and *F. falciforme*) showed very different colors. The outline of the mycelium as described by Ainsworth and Bisby's (1971) was found between species at day 10 of culture.

The microscopic characters studied (dimension, morphology and sporulation) also revealed differences among species. The frequency of these forms shows that *Fusarium* spp. produced sickle-shaped macroconidia in proportion of 90.5% (*F. stilboides*) and of all other curved shape with a proportion of 94% (*F. xylarioides*). A small proportion of the form is filed by the Fusoid *F. stilboides* (9.5%). However, observations under a microscope focused on traits such as sporulation, conidial size, the number of partition and conidial shape. By basing ourselves on the density of sporulation macroconidia and

microconidia which varied depending on the species, the difference in size of conidia based on genetics was more important. This was confirmed by Houssiau (2004). The measurements found these macroconidia and microconidia are consistent with studies by Saccas 1950. The macroconidia were characterized by the absence or presence of one wall in the vegetative stage as described by Booth (1971). By cons microconidia are devoid of wall. This was confirmed by Tshilenge et al (2004). Based on conidial morphology, species have submitted forms such as inspired by Saccas (1951).

We note that following this study, conducted to characterize the different *Fusarium* species associated with the decline of coffee in the Democratic Republic of Congo, it appeared that the *F. xylarioides* reflecting a more critical that *F. solani, F. stilboides*, *F. falciforme* and *F. lateritium*. At each level of characterization, significant differences were indicated between discrimination *F. xylarioides* and other *Fusarium* spp.

Bearing in mind the factors influencing the expression of a disease, namely, the pathogen, the host and the environment, this study is in terms of knowledge of the pathogen. The results obtained and the *Fusarium* spp. exhibit plasticity for both mycelial growth, pigmentation and contour mycelium for sporulation, the dimension and morphology on culture media. Characterization of *Fusarium* spp in culture is virtually an analytical macroscopic and microscopic quick but has limitations because of overlap of some values that can not determine clearly the difference between species. For a better characterization it is important to consider other criteria such as molecular characterization.

However, given the variability in cultural characters and symptoms observed in the field, it is imperative to establish a relationship between the variability in observed characteristics and pathogenicity of *Fusarium* spp. to determine the precise role of different *Fusarium* spp. in the resurgence of CWD. The studies and researches that are running could be promising, but given the current state of the plantations and the spread of CWD in the DRC, of short and medium term should be undertaken. The magnitude of the disaster will involve the intervention of the political and administrative authority for their success. We believe in a rigorous and systematic destruction by fire of plantations greatly impaired. The role of this authority would be to compensate farmers and give them the option of converting food crops to break the life cycle of the pathogen. A similar solution in the short and medium term was proposed by Pochet (1988) and has been beneficially used in Côte d'Ivoire (Meiffren, 1961).

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