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Field Assessment of the Potential Role of *Fusarium* Species in the Pathogenesis of Coffee Wilt Disease in Democratic Republic of Congo

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Authors' contributions

The present study was carried out in collaboration between all authors. Authors PTD and AKM designed, wrote the protocol and monitored the field trial. Author KKM conducted the field trial and wrote the first manuscript in collaboration with author LTL. Authors ANN and MMM completed the data analysis and the literature review. Author MMM wrote the final manuscript. All authors read, corrected and approved the final manuscript.

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ABSTRACT

Aim: To determine the probable role of 3 species of *Fusarium* (*F. falciforme, F. solani* and *F. stilboides*) in the pathogenesis of Coffee Wilt Disease (CWD).
Study Design: The field trial was performed using a Randomized Completed Block Design (RCBD) replicates three times.

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Place and Duration of the Study: The study was conduct in the Experimental Garden of Department of Biology, Faculty of Sciences, University of Kinshasa in Democratic Republic of Congo, between November 2005 and February 2006.
Methodology: Four *Fusarium* species (*F. falciforme, F. solani, F. stilboides* and *F. xylarioides*) were inoculated alone, or the first three strains were inoculated in combination with *F. xylarioides* in coffee seedlings 10 months old. Observations were focused on the time of expression of main CWD symptoms, the rate of each symptom observed, and the presence of each pathogen in dead woods of inoculated seedlings.
Results: Results obtained showed that all *Fusarium* species induced main symptoms of CWD at varying moment and degrees. Chronologically, leaf yellowing appeared an average 25 dai, followed by leaf browning (46 dai), leaf drying (61 dai), mortality (75 dai) and defoliation (77 dai). Significant differences (*P = .05*) were observed between treatments considered. All *Fusarium* induced seedlings mortality and were isolated in dead woods at 1 – 4 cm from the point of inoculation. In general, the search of synergistic interaction between *Fusarium* spp. inoculated in combination with *F. xylarioides* showed enhanced ability to induce various symptoms of CWD when species are used together.
Conclusion: The present study demonstrates that CWD could be reconsidered as a parasitic complex, and in natural conditions all *Fusarium* species used present a danger for coffee growing.

Keywords: Coffee wilt disease; Fusarium species; pathogenesis; DR-Congo.

1. INTRODUCTION

In Democratic Republic of Congo (DRC), coffee (*Coffea* spp.) is one of the key cash crops for small farmers, and represents an important commodity in the economy of the country [1]. The coffee species grown in DRC are *C. arabica*, *C. canephora* var. *robusta* and *C. canephora* var. *kouillou.* Among them, *Robusta* coffee is a paramount economic importance; it represents almost 90% of all the plantations across the country. It is the third exported product and generates nearly 9% of the total revenue from exportation for the country [2]. Statistical estimates report that coffee export represents 60-65% of the total value of exported agricultural exports [1].

However, since the 90s, coffee production in DRC faced a remarkable chute whose causes are varied. The main causes include the aging and the poor management of plantations, the degeneration of the existing planting material, the lack of skills and knowledge of coffee production by smallholder producers [2], the coffee price decline in the international market, and the presence of various diseases. Among the coffee diseases, Coffee Wilt Disease (CWD) represents the most devastating of coffee plantations in the major coffee producing regions in Eastern and North Kivu provinces of DRC [2,3]. CWD is a fungal disease caused by *Gibberella* (*Fusarium*) *xylarioides* Heim & Saccas [4–7] which is manifested by yellowing and wilting of leaves, followed by the defoliation and drying of the crown. At the final stage, the entire plant withers and dies [5]. The characteristic symptom of this disease is the dark aspect of the subcortical zone of the base of the trunk [8].

The struggle against this disease is and remains the mechanical control. It consists of picking, cutting and incineration *in situ* of infected plants as soon as they were identified. This practice, although the basis for the reduction of infectivity of *F. xylarioides* has shown its limits. Among the root causes of the inefficiency of this form of struggle, there is the onerous task of removal diseased plants, negligence in the application of phytosanitary measures, the abandonment in field of debris of diseased plants and the desire not remove the infected plants carry cherries. These factors are probably the basis for the resurgence of the disease in some coffee plantations. Many studies were conducted on the pathogenesis of CWD. Isolations made by Girma [9] and Tshilenge-Djim et al. [10] on infected woods of coffee trees shown the presence of other *Fusarium* species associated with *F. xylarioides*. However, the role of these *Fusarium* species in the pathogenesis of CWD remains uncertain. It is in this context that this study is aimed to determine the probable role of *F. falciforme, F. solani* and *F. stilboides* in the pathogenesis of CWD.

2. MATERIALS AND METHODS

2.1 Experimental Field Location

The study was conducted in the Experimental Garden of the Department of Biology, Faculty of

Sciences, University of Kinshasa, DRC. The geographic coordinates recorded with the GPS (extrex Summit Garmin) indicated 4°19'S latitude, 15°8'E longitude, and 330 m of altitude. Data reported in Makoko et al. [11] indicate that experimental site falls within the Aw4 climate type according to Köppen classification characterization with 4 months of dry season (from second mid-may to first mid-september) coupled with 8 months of rainy season (from second mid-september to first mid-may) sometimes interrupted by a short dry season in January/February. Daily temperature averages 24.5°C and accuses small variations, and the annual rainfall is close to 1500 mm. The July and February are respectively the coldest and warmest month. The relative humidity is highest in April and May, and is minimum in September and October.

Data related to climatic conditions prevailing during the field trial are reported in Table 1. Climatic conditions were favorable for the development of *Fusarium* species. Indeed, for its development, fungus usually requires temperatures between 10 - 40°C, and humidity prevailing during the field trial was favorable. The rainy season (November and December) corresponding to the period of the intense flow of raw sap was favorable to the growth of the fungus, and promotes the transport of the fungus in the vessels of plant.

2.2 Plant Material Used

Coffee seedlings used were 10 months old, obtained from seeds of *Coffea canephora* var *robusta* harvested in Beni region, eastern DRC. The seeds were sown in hotbed on January 18th 2005, and seedlings transplanted ten months later into polyethylene bags of 15 cm x 35 cm x 0.05 cm filled with soil from the valley of the Monastery Prieuré Notre Dame de l'Assomption. The soil used was characterized by black coloration according to Munsell scale [12], high porosity and a pH of 5.1. No fertilizer was used, and seedlings were watered every two days during dry periods.

2.3 *Fusarium* Strains Used

All *Fusarium* strains used (Table 2) provided from infected coffee trees collected in DRC; they were isolated and purified in the Unit of Phytopathology laboratory (Université Catholique de Louvain, Belgium), where they are stored in the fungus collection. These strains are stored as parent-strains in test tubes containing the synthetic nutrient agar (SNA) medium under paraffin. The SNA medium used was constituted of: KH_2P0_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 g; Agar Merck®: 20 g; and distilled H_2O: 1000 ml. The *Fusarium* strains were first taken to their revival by transplanting streaked on SNA medium in Petri dishes. The Petri dishes were returned, labeled and tightly closed with parafilm, and then incubated for 10 days at laboratory temperature (± 27°C). The subcultures obtained from strains-girls were used to produce inoculum of each strain. The inoculum used consisted to a pellet of 5 mm in diameter cut with a sterile scalpel blade outskirts of the mycelium of strains-girls previously obtained on SNA. The inoculum of each strain has been taken where high concentration of conidia was previously observed upside of the Petri dishes under a microscope (Olympus BX 40).

Table 1. Averages temperature, relative humidity and rainfall prevailing during the field trial

Month	Temperature (°C)	Relative humidity (%)	Rainfall (mm)
November (2005)	28.6	81.3	248.7
December (2005)	27.7	89.6	266.6
January (2006)	30.5	98.9	24.0
February (2006)	26.7	81.4	27.5

Table 2. Different *Fusarium* species used in the study

Strain	Species	Origin	Isolated at
MUCL45592	*F. xylarioides*	Isiro, DRC	UCL
MUCL45428	*F. stilboides*	Butembo, DRC	UCL
MUCL46055	*F. solani*	Yangambi, DRC	UCL
MUCL43880	*F. falciforme*	Bas-Congo, DRC	UCL

2.4 Technique of Inoculation

The seedlings inoculation was made using the method of incision (wound). The stem of seedlings was first superficially disinfected with 70% ethanol which is allowed to evaporate for 10 minutes. The inoculation was done by insertion of the inoculum into an incision at the base of the stem. The incision was made using a sterile scalpel blade at 2 – 3 cm above the collar of seedling in the plane of the first pair of true leaves, and inoculum was maintained in place by a ligature made with parafilm. Each strain of *Fusarium* was inoculated alone or in combination with *F. xylarioides*. Each inoculation made represented a treatment, and was as follows: *F. falciforme* (Ff); *F. solani* (Fs); *F. stilboides* (Fst); *F. xylarioides* (Fx); *F. xylarioides* + *F. falciforme* (Fx + Ff); *F. xylarioides* + *F. solani* (Fx + Fs); *F. xylarioides* + *F. stilboides* (Fx + Fst); and control (un-inoculated seedlings). In case of combination, inocula of two strains to combine were inserted in the same incision.

2.5 Data Recorded, Experimental Design and Statistical Analysis

Observations were focused on the time (number of days) between inoculation and the expression of different CWD symptoms described by Tshilenge-Djim [10], such as yellowing, wilt, browning and drying of leaves, defoliation and seedlings mortality. Observations were made every two weeks. The yellowing of leaves was characterized by leaves discoloration which turn from green to yellow. The wilting was characterized by leaves which become flaccid and tend to look down the stem following the loss of turgor. The browning was appreciated by the change in leaf coloration which turn to dark brown or presenting at the same time a wet appearance or sometimes more or less oily. The drying was appreciated by the brittleness of leaves to the touch. In addition, the rate of each parameter was noted. The seedlings mortality was appreciated by the percentage of seedlings died compared to seedlings inoculated. The mortality was confirmed by the complete drying of the seedling. The defoliation was determined by the percentage of fallen leaves compared to leaves produced. At the end of the trial, the re-isolation of pathogens was made on died coffee seedlings to check Koch's postulate.

The field trial was conducted using a randomized complete block design (RCBD) with three replications. Each plot has 12 coffee seedlings. Data recorded were submitted to analysis of variance (ANOVA) using R (R-2.12.0) software. Means comparison was performed by LSD test at 5% of probability level.

3. RESULTS

Data related to the interval of time (days) between inoculation and expression of symptoms of CWD are reported in Table 3. Results related to the rate of symptoms of CWD are presented in Table 4. Results of the re-isolation of *Fusarium* strains are reported in Table 5.

3.1 Assessment of the Pathogenicity of *Fusarium* Species Used

Analysis of data reported in Table 3 shows that all *Fusarium* species used expressed main symptoms of CWD. In general, the time duration between coffee seedlings inoculation and symptoms expression varies according to strains. Except for defoliation, statistical analysis showed significant difference for other parameters studied. Chronologically, leaf yellowing was first

Table 3. Interval of time (days) between inoculation of coffee seedlings and the expression of CWD symptoms

Strains	Yellowing of leaves	Browning of leaves	Drying of leaves	Defoliation	Seedlings mortality
F. falciforme	28.0 ± 3.5	53.9 ± 3.1	67.4 ± 1.5	76.2 ± 1.9	72.0 ± 2.1
F. solani	27.5 ± 1.5	50.0 ± 2.5	66 ± 1.7	77.2 ± 1.5	70.7 ± 1. 3
F. stilboides	22.7 ± 2.3	44.5 ± 3.4	68.8 ± 2.5	78.5 ± 2.5	79 ± 1. 2
F. xylarioides	24.3 ± 1.1	43.2 ± 2.4	57.2 ± 2.4	77.2 ± 2.6	78.2 ± 2.5
Fx + Ff	27 ± 2.2	42.7 ± 1.8	54.5 ± 3.5	77.5 ± 2.4	75.5 ± 3. 2
Fx + Fs	21.2 ± 2.1	37.7 ± 2.0	54.3 ± 1.5	76.0 ± 1.5	75.5 ± 2.4
Fx + Fst	22.7 ± 3.5	49.2 ± 1.5	56.2 ± 1.4	76.7 ± 2.4	74.5 ± 2.1
Control	N.O.	N.O.	N.O.	73.9 ± 3.3	N.O.
$LSD_{0.05}$	3.8	5.7	4.6	N.S	6.7

N.S: Not significant

Table 4. Rate (%) of different CWD symptoms recorded on coffee seedlings inoculated with *Fusarium* species alone and in combination

Strains	Yellowing of leaves	Browning of leaves	Drying of leaves	Defoliation	Seedlings mortality
F. falciforme	24.7 ± 1.7	29.0 ± 2.8	30.5 ± 2.2	8.3 ± 0.9	10.3 ± 2 .2
F. solani	24.4 ± 1.5	27.0 ± 1.8	2.7 ± 0.5	8.3 ± 0.9	8.3 ± 0.8
F. stilboides	18.7 ± 2.1	28.6 ± 1.9	8.3 ± 2.3	12.4 ± 2.4	16.3 ± 2 .3
F. xylarioides	18.7 ± 2.1	24.7 ± 2.3	11 ± 2.5	12.4 ± 2.4	18.7 ± 2. 5
Fx + Ff	20.8 ± 2.5	30.9 ± 2.5	11.1 ± 2.2	10.4 ± 2.1	12.4 ± 1.5
Fx + Fs	22.9 ± 2.2	20.7 ± 2.4	8.3 ± 1.3	12.4 ± 0	10.3 ±1.4
Fx + Fst	16.9 ± 1.4	24.9 ± 2.2	13.8 ± 2.4	16.6 ± 2.5	24.9 ± 2.2
Control	0	0	0	9.5 ± 3.3	0

observed, followed by browning, drying, defoliation and seedlings mortality. The yellowing was recorded an average 21 days after inoculation (dai) on seedlings inoculated with *F. xylarioides* + *F. solani*, while it was observed 28 dai on seedlings inoculated with *F. falciforme*. The leaf browning was recorded an average 38 dai on seedlings inoculated with *F. xylarioides* + *F. solani*, while it was recorded 54 dai on seedlings inoculated with *F. falciforme*. The drying was recorded an average 54 dai on seedlings inoculated with *F. xylarioides* + *F. solani*, while it was noted 69 dai on seedlings inoculated with *F. stilboides*. The defoliation was recorded 76 dai on seedlings inoculated with *F. xylarioides* + *F. solani*, and 79 dai on seedlings inoculated with *F. stilboides*. Although control seedlings have not been inoculated, they expressed defoliation which was recorded an average at 74th day of observation. The seedlings mortality was recorded 71 dai on seedlings inoculated with *F. solani,* while it was noted 79 dai on seedlings inoculated with *F. stilboides*.

Results reported in Table 4 show that the percentage of inoculated seedlings expressing CWD symptoms varies significantly (*P = .05*) from each strain to another. The lowest rate of leaf yellowing (17%) was recorded on seedlings inoculated with *F. xylarioides* + *F. stilboides*, while the highest rate (25%) was noted on seedlings inoculated with *F. falciforme*. Seedlings inoculated with *F. xylarioides* + *F. solani* expressed the lowest rate of leaf browning (21%), while those inoculated with *F. xylarioides* + *F. falciforme* showed the highest rate (31%). The lowest rate of leaf drying (3%) was recorded on seedlings inoculated with *F. solani*, while the highest rate (31%) was noted on seedlings inoculated with *F. falciforme*. Seedlings inoculated with *F. falciforme*, and *F. solani* expressed the lowest rate (8%) of defoliation, while seedlings inoculated with *F. xylarioides* + *F. stilboides* showed the highest rate (17%). The lowest mortality rate (8%) was noted on seedlings inoculated with *F. solani*, while the highest mortality rate (25%) was recorded on seedlings inoculated with *F. xylarioides* + *F. stilboides*.

Table 5. Presence/absence of *Fusarium* species in dead woods of coffee seedlings

Strains	Presence (+) or absence (-) of *Fusarium* species in dead woods	Isolation distance from the point of inoculation (cm)
F. falciforme	+	± 1
F. solani	+	± 1
F. stilboides	+	± 2
F. xylarioides	+	± 4
Fx + Ff	+ -	± 4
Fx + Fs	+ -	± 4
Fx + Fst	+ +	± 2
Control	*	*

** Not made*

3.2 Re-isolation of *Fusarium* Species

The re-isolation of *Fusarium* strains was made from the inoculated seedlings showing complete drying. Results obtained show that all *Fusarium* strains inoculated alone were re-isolated on dead woods, while in cases of combined strains, *F. xylarioides* and *F. stilboides* were both re-isolated together, and *F. xylarioides* was re-isolated alone where its inoculation was made with *F. solani* or *F. falciforme*. These different strains were found not only in the inoculation area, but in areas more or less distant from the point of inoculation.

4. DISCUSSION

Coffee Wilt Disease (CWD) constitutes one of the major constraints of coffee production in most African countries [6,7,13,14]. The present study shows that all *Fusarium* strains used induced leaf yellowing, browning, drying, defoliation and seedlings mortality, which are described as characteristic symptoms of CWD. The wilting was not observed on all inoculated seedlings; this may be due to the relative humidity (mean value: 87.8%, Table 1) demonstrating the drying capacity of the air. These findings mean that under certain environmental conditions, wilting or other foliar symptom may or not appear on coffee plants infected with CWD. In general, chronologically, leaf yellowing appeared an average 25 dai, followed by leaf browning (46 dai), leaf drying (61 dai), mortality (75 dai) and defoliation (77 dai) (Table 3). This observation is consistent with that done by Tshilenge-Djim et al. [14] who mentioned that CWD symptoms are varied both in their nature and their chronological sequence from the time of their appearance.

In general, the search for synergistic interaction between various *Fusarium* spp. inoculated in combination with *F. xylarioides*, the main causative species of CWD, showed enhanced ability to induce various symptoms of CWD when these species are used together. According to results reported in Table 3, it appeared that the time of onset of leaf yellowing, browning and drying, was shorter in the case of composite inoculation between *F. xylarioides* and *F. solani*. The same stimulating effect of *F. xylarioides* by *F. falciforme* and *F. solani* also seems to be apparent on the rate of yellowing (Table 4). It appears that the rate of yellowing induced by *F. xylarioides* alone was 19%, and increased to 21 and 23% when *F. xylarioides* is associated with *F. falciforme* and *F. solani*, respectively. The rate of leaf browning also shows synergy in the association between the *F. falciforme* and *F. xylarioides*. It was registered an increase in rate of leaf browning to 31%, whereas with the two species separately inoculated, the rate of browning recorded was 29 and 25%, respectively (Table 4).

As for mortality, the time of expression and the rate of this parameter on inoculated seedlings were strengthened by the combination of *F. xylarioides* and *F. stilboides*. The inoculated seedlings with both species showed faster the case of mortality (75 dai) (Table 3) and higher rate (25%) (Table 4). The same trend of synergism was observed with the rate of defoliation for which, it has been observed that the seedlings inoculated with *F. xylarioides* and *F. stilboides* in combination, presented higher value (17%) (Table 4). The present findings are consistent to results reported in Uganda by Serani et al. [15] who have shown that, *Fusarium* species usually isolated on *robusta* coffee trees, including *F. solani*, *F. stilboides* and *F. xylarioides* could produce at varying degrees' symptoms of CWD on inoculated coffee plants. In the present study, the defoliation has a significant variability, and can not be used as a criterion for assessing the pathogenicity of *Fusarium* species. In effect, the defoliation was observed as on the control seedlings than those inoculated.

Results reported in Table 5 show that all *Fusarium* species used are responsible of seedlings mortality, and presented at the same time similar symptoms to those observed on seedlings infected with *F. xylarioides*. They were re-isolated in cases of single inoculation, while in combination, only *F. xylarioides* and *F. stilboides* were both isolated together, or *F. xylarioides* was isolated alone. The *F. xylarioides* was isolated at places localized at ± 4 cm from the point of inoculation, while all others species were isolated at 1 – 2 cm from the place of inoculation. This difference in location did not prevent those *Fusarium* species also cause leaf symptoms and mortality. In contrast, a previous study conducted in the conditions of confinement by Tshilenge-Djim et al. [10] has shown that other *Fusarium* species are accompanying *F. xylarioides*, but their role in the expression of the infection was not evident.

5. CONCLUSION

The present study demonstrated that all *Fusarium* species used induced main symptoms of CWD at varying moment and degrees. Results obtained show that CWD could be reconsidered as a parasitic complex, and are likely to challenge the coffee farmers on the danger of all *Fusarium* species in natural conditions. Thus, a new pest management approach of this disease will be oriented to the research of plant material with broad and durable resistance to these *Fusarium* species. They will appear in the artificial inoculation program in screening assays, next to *F. xylarioides*. A selection of resistant materials based on reactions of genotypes to a wide range of pathogens seems more durable and suitable in the case of a parasitic complex.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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