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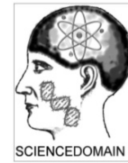

Cultural, Morphological and Pathogenic Variability among *Fusarium oxysporum* f. sp. *phaseoli* Causing Wilt in French Bean (*Phaseolus vulgaris* L.)

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Authors' contributions

This work was carried out in collaboration between all authors. Author PKM carried out the experiments, performed the statistical analysis, managed the literature searches and wrote the first draft of the manuscript. Authors PMW, SAO and JWK designed and supervised the study and reviewed the draft. All authors read and approved the final manuscript.

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ABSTRACT

Aim: This study aimed at evaluating cultural, morphological and pathogenic variability among *Fusarium oxysporum* f. sp. *phaseoli* strains isolated from French beans.
Study Design: Cross-sectional study.
Methodology: The French beans showing *Fusarium* wilt symptoms were obtained from different fields in Kabaa irrigation scheme in Machakos County, Kenya. The diseased plants were washed and cut into 5 mm pieces which were surface sterilized before plating on *Fusarium*-selective medium. They were incubated for 10 days at 25-26℃. The developing colonies were transferred on Potato Dextrose Agar (PDA), Carnation Leaf Agar (CLA) and Spezieller Nahrstoffarmer Agar (SNA) media for cultural and morphological characterization. Pathogenicity was assessed using on French bean 'Amy' variety.
Results: From 84 *Fusarium* isolates obtained, 18 were confirmed to be *F. oxysporum* from which 8 were confirmed to be *F. oxysporum* f. sp. *phaseoli (Fop).* Variations existed cultural and

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morphological variations among the 8 isolates. Isolates showed luxuriant, moderately luxuriant and scanty aerial mycelial growth. Mycelial texture was either fluffy or fibrous. Isolate *Fop*8 had the highest growth at 85 mm at 7th day followed by *Fop*3 (84 mm) and *Fop*6 (84 mm) while *Fop*7 had the least growth at 71 mm. The colony colour was purple, pink or white. Microconidia sizes ranged from 8 X 3.0 to 10 X 3.4 µm while macroconidia ranged from 28 X 3.8 to 42 X 4.2 µm. Macroconidia had 3 septa. The microconidia were abundant and aseptate. Chlamydospore formation was terminal and intercalary, occurring in singles and in pairs. Although all the 8 isolates were pathogenic on 'Amy' French bean variety, their pathogenic potential was significantly different ($P < 0.01$). The most pathogenic isolate was *Fop*03, followed by *Fop*06 and *Fop*07 at means of 97.0, 92.4 and 92.0%, respectively. The least pathogenic isolate was *Fop*05 with a mean of 65.9% pathogenicity.

Conclusion: The isolates in this study are culturally and morphologically varied and have high pathogenicity on French bean.

Keywords: Conidia; French bean; Fusarium oxysporum f. sp. phaseoli; Fusarium wilt.

1. INTRODUCTION

Fusarium oxysporum f. sp. *phaseoli ((Fop)* W.C. Snyder & H. N. Hans*)* is an important pathogen that causes of Fusarium wilt in French bean (*Phaseolus vulgaris* L.). Losses of up to 100% have been reported. The disease is one of the most devastating diseases of French bean worldwide [1]. The wilt pathogen was first described in United States in 1929 by Harter [2]. The fungal wilt pathogens are primarily found in the soil but are able to infect and block the vascular system of crops resulting into wilting [3]. Most strains of the wilt fungi are not pathogenic but lives as saprophytes. However, the pathogenic strains are responsible for serious diseases that destroy the vascular system reducing crop productivity [4]. French bean crop losses of up to 100% have been reported [5]. *Fop* strains show diversity in terms of their cultural, morphological and pathogenic characteristics. The identification of this fungus is generally based on these features. Cultural features used in characterization include mycelial growth, texture and colour while the morphological ones are conidial measurements, septations and chlamydospore formation [6]. The culture may vary from white to purple while growth may vary from luxuriant to scanty on aerial mycelia, fluffy and fibrous based on mycelial texture and long, medium and short macroconidial length. Pathogenicity also varies with strain with some being mildly pathogenic to some that are severely [7].

French bean is a crop with great potential for addressing food insecurity, income generation, foreign exchange earner and poverty alleviation in Kenya [8]. This crop ranks first among vegetables produced for the export market in Kenya [1,9]. However, its production is greatly hampered by *Fusarium* wilt disease. The disease is becoming increasingly important due to its rising levels associated with enhanced spread of the pathogen related to increased mechanization in field activities, repeated planting of similar crop in the same area and production in regular continuous cycles within a single year [10].

Fusarium wilt pathogen can survive in soil for extended periods in the absence of the host, mainly in the form of thick walled chlamydospores [6]. These spores are resistant to adverse conditions like extreme temperature, chemicals and dehydration. This makes this fungus very persistent in the soil. Indeed, once an area becomes infected with *F. oxysporum*, it usually remains so indefinitely [6]. The fungus is not easy to control. The proximity of the host roots induces the dormant propagules of the pathogen to germinate and initiate infection. The pathogen can spread wide geographical distances through seeds [10]. After spore germination, infection hyphae adhere to the host roots and penetrate them directly. The mycelium then advances intercellularly through the root cortex until it reaches the xylem vessels and enters them through the pits. At this point, the fungus switches to a highly peculiar mode of infection, during which it remains exclusively within the xylem vessels, using them as avenues to readily colonize the host [11]. This is mainly accomplished by the production of microconidia, which are detached and carried upward in the sap stream. The microconidia eventually germinate and the mycelium penetrates the upper wall of the vessels producing more microconidia in the next vessel. Yellowing and senescence of mature leaves occurs. Chlorosis

progresses throughout the plant causing the entire foliage to be bright yellow, wilted and discoloured. Bean plants are completely stunted if infected when young. The vascular tissues remain reddish [6].

The characteristic wilt symptoms appear as a result of severe water stress, mainly due to vessel clogging. Wilting is most likely caused by a combination of pathogen activities such as the accumulation of fungal mycelium and/or toxin production and host defense responses, including production of gels, gums and tyloses and vessel crushing by proliferation of adjacent parenchyma cells [12]. Serious infections kill the crop in few weeks. As long as the plant is alive, the vascular wilt fungus remains strictly limited to the xylem tissues and a few surrounding cells. Only when the infected plant is killed by the disease does the fungus invade the parenchymatous tissue and sporulate profusely on the plant surface. *F. oxysporum* thus occupies a highly specific ecological niche, shared by only a few other fungal plant pathogens such as *Verticillum dahlia* and *Ceratocystisulmi* [7].

Fusarium wilt disease is a serious constraint to production of this crop [1]. With intensive use of fungicides to increase production of this crop, there is growing concern of evolution of *Fop* strains that are highly pathogenic and resistance to fungicides [13] giving rise to more aggressive and virulent strains. *Fusarium* wilt is prevalent in most French bean producing areas and losses due to the disease have been gradually rising in Kenya [9]. *Fop* strains show variability in their cultural, morphological and pathogenic characteristics. There is need to further the understanding of this pathogen for better management and control in French bean. Therefore this study aimed at assessing the cultural, morphological and pathogenic variability of *Fop* isolates from Kabaa irrigation scheme in Machakos County, a French bean growing region in Kenya.

2. MATERIALS AND METHODS

2.1 Sampling, Fungal Isolation and Characterization

An extensive field survey was carried out in Kabaa irrigation scheme in Machakos County, Kenya where French bean is grown extensively during the month of March 2015. Ten fields were identified which had crops showing *Fusarium* wilt symptoms. From each of the field, five random microplots each measuring 5 x 5 m^2 were identified. Five French bean plants showing characteristic *Fusarium* wilt symptoms were collected from each microplot, placed in paper bags, brought to the Mycology laboratories of University of Nairobi where they were stored at 4℃.

From each diseased plant, the stems and roots were washed under running water and cut into 5 mm pieces. These pieces were surface sterilized with 0.5% sodium hypochlorite solution for 5 minutes and rinsed twice with sterilized distilled water. The pieces were then dried with sterile filter paper and directly plated (two to three pieces per plate) on *Fusarium* specific Pentachloronitrobenzene-Peptone Agar (PPA) medium (Fig. 1) and incubated for 10 days at 25-26℃. This medium is *Fusarium*-selective.

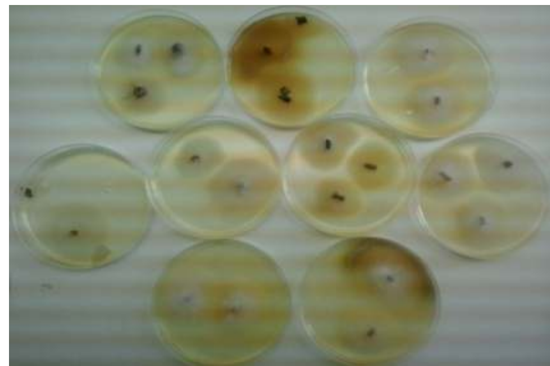

Fig. 1. Plant pieces from diseased crops plated on PPA media after 7 day of incubation

Fusarium growing on PPA medium plates were sub-cultured on Spezieller Nahrstoffarmer Agar (SNA) media and incubated at 25-26℃ for 3 days from which cultures were plated on 2% Tap Water Agar (TWA) medium. Single spores germinating on 2% TWA were plated on Potato Dextrose Agar (PDA), SNA and Carnation Leaf Agar (CLA) and incubated for 7 days at 25-26℃ for growth and identification. Colonies exhibiting taxonomic features of *Fop* were identified according to Nelson et al. [14] and Leslie and Summerell [15]. Morphological identification was based on characteristics of the macroconidia, phialides, microconidia, chlamydospores and colony growth traits. Pure cultures of all the isolates were preserved on SNA at 4℃. Cultural and morphological characters were assessed on 7 day old cultures. Colony growth rate, pigmentation, aerial growth and texture were evaluated on cultures grown on PDA. Microconidia characteristics were assessed on

cultures grown on SNA while those of macroconidia were assessed on cultures grown on CLA.

2.2 Pathogenicity Testing of Isolates

Pathogenicity tests were done in a greenhouse at University of Nairobi (Chiromo campus) using French bean cultivar "Amy" to evaluate the infective ability of isolated strains as described by Perveen et al. [16].

2.2.1 Inoculum preparation

Each isolate was grown on potato dextrose agar (PDA) for 10 days at room temperature (25 ± 2℃) under 24 h fluorescent lights. Spore suspensions for inoculation were prepared by flooding cultures with sterile distilled water, dislodging conidia with a disposable hockey stick afterwards, filtering through sterile cheese cloth to avoid agar residues, and adding water to obtain a final volume of 100 ml. Spores were counted on a hemocytometer. The spore concentration was adjusted to 10^6 conidia /ml by adding sterile distilled water. Inoculum of each *Fusarium* isolate was prepared by adding 2 ml of spore suspension into a bag containing previously prepared inoculum media. Inoculum media was prepared using sorghum grains. Sorghum grains were washed in tap water 3 times and soaked overnight in hot tap water. After draining excess water, 50 g of sorghum grains were placed in each jar and autoclaved for 60 min at 121℃ on two consecutive days. After cooling, each jar was inoculated with 2 ml of the spore suspension of different isolates that had been previously prepared. Jars were then shaken every day for approximately one minute and incubated under constant fluorescent light at room temperature (25 ± 2℃) during 3 weeks. The colonized sorghum grains were then dried under a laminar flow hood for 2 days, ground and stored in bags at 4℃.

2.2.2 Planting

French bean "Amy" variety seeds (Simlaw seeds) were surface-sterilized by dipping the seeds in 1% sodium hypochlorite solution for 3 minutes, and then rinsed several times with sterilized distilled water. Each sterilized pot was filled with a mixture of 500 g of autoclaved soil:sand:compost mixture (1:1:1). The media was sterilized at 121℃ for 2 consecutive days. For each isolate, 10 g of inoculum media were introduced in the pot mixture. The set up was left for 1 week before planting was done. Four French bean seeds were sown in each pot. Pots without pathogen inoculation served as control. There were three replicates per each treatment set in a completely randomized block design. Plants were irrigated with sterile water and observed daily to record disease symptoms. Disease severity was measured according to Perveen et al. [16], from 2 week of planting up to 45 days of growth. Symptoms were recorded according to a scale ranging from 1 to 5 (1- No symptoms; 2- 25%, wilting/yellowing of the plant; 3- 50%, wilting/yellowing of the plant; 4- 75%, wilting/yellowing of the plant; 5- Death of the plant).

3. RESULTS

3.1 Cultural and Morphological Variations

From the diseased French bean plants, total of 84 *Fusarium* isolates were obtained, of which only 18 were confirmed to be *F. oxysporum* while the rest were *F. solani.* The 18 *F. oxysporum* isolates were further characterized into 8 *F. oxysporum* f. sp. *phaseoli* isolates. These isolates varied in their cultural and morphological characteristics. The isolates showed luxuriant, moderately luxuriant and scanty aerial mycelial growth (Table 1). The colony diameter for the isolates at 7th day of growth differed among the isolates incubated at 25-26℃ on PDA (Table 1). The diameter ranged from 71 to 85 mm in 90 mm Petri plates. *Fop*8 had the highest growth (85 mm), followed by *Fop*3 (84 mm) and *Fop*6 (84 mm) isolates. Isolate *Fop*2 had the least growth at 71 mm (Table 1). Based on mycelial growth texture, two groups of isolates were obtained; fluffy and fibrous growth (Table 1). The pigmentation of the isolates on PDA medium varied from white, pink to purple. Fig. 2 shows the pure cultures of different *Fusarium oxysporum* f. sp. *phaseoli* isolates on the 5th day of growth on PDA. The rate of sporulation was varied among the isolates. Isolates *Fop*1, *Fop*3, *Fop*6, *Fop*7 and *Fop*8 had profuse sporulation while isolates *Fop*2, *Fop*4 and *Fop*5 had moderate sporulation (Table 1).

The isolates differed in their micro and macroconidia in terms of size and number of septa. The length X breadth of the microconidia ranged from 8 X 3.0 to 10 X 3.4 µm with a mean size of 9 X 3.4 µm (Table 2). Macroconidia varied between 28 X 3.8 to 42 X 4.2 µm with a mean of 37 x 3.3 µm (Table 2). All the isolates had 3 septa macroconidia. The macroconidia of all the isolates were slightly sickle-shaped with slightly

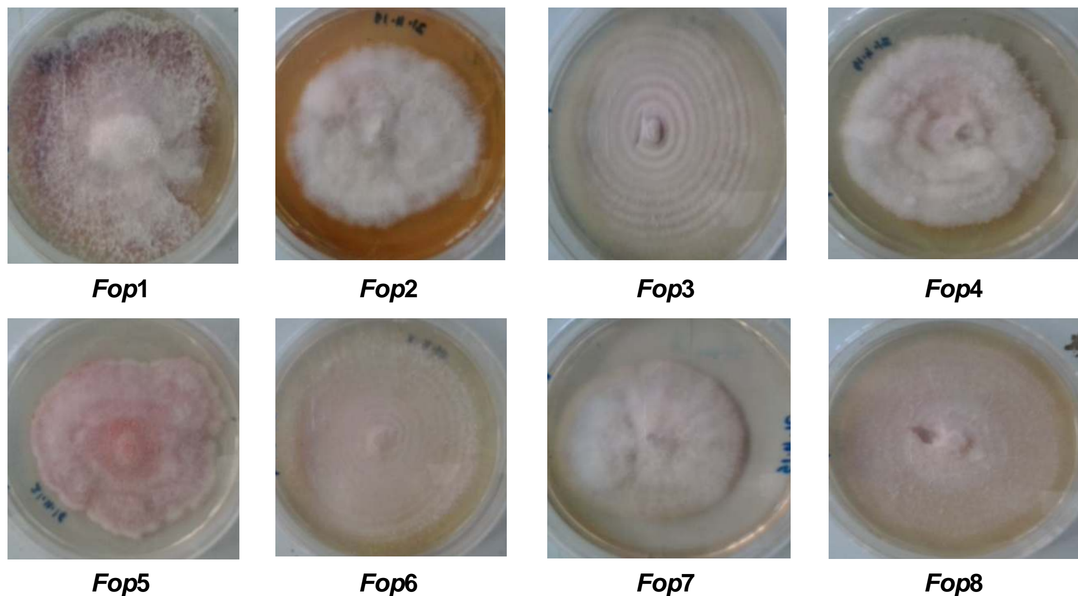

Fig. 2. Different *Fusarium oxysporum* f. sp. *phaseoli* isolates on the 5th day of growth on PDA

foot shaped basal cell and attenuated apical cells. The microconidia produced were abundant and aseptate. They were borne by short monophialides. Majority were oval in shape while a few were reniform in shape. Fig. 3 shows microconidia of *Fop*3.

The chlamydospores were present at the terminal or intercalary positions, usually in singles and in pairs. In all the isolates, the chlamydospores had smooth walls. The results indicated that sporulation varied among the isolates. The isolates exhibited a high level of diversity in terms of cultural and morphological characteristics on PDA, SNA and CLA media.

Fig. 3. Microconidia of *Fop*3 isolate

Table 1. Cultural characteristics of *Fusarium oxysporum* f. sp. *phaseoli* isolates

Isolate	Aerial mycelium	Mycelia texture	Mycelia colour	Colony diameter at 7th day (mm)	Sporulation
*Fop*1	Luxuriant suppressed	Fibrous	Purple	80	Profuse
*Fop*2	Scanty, suppressed	Fibrous	White	73	Moderate
*Fop*3	Luxuriant, suppressed	Fluffy	White	84	Profuse
*Fop*4	Luxuriant, suppressed	Fluffy	White	74	Moderate
*Fop*5	Luxuriant, suppressed	Fluffy	Pink	72	Moderate
*Fop*6	Luxuriant	Fibrous	White	84	Profuse
*Fop*7	Moderate luxuriant	Fibrous	White	71	Profuse
*Fop*8	Luxuriant	Fluffy	White	85	Profuse
Mean				78	

Table 2. Microconidia and macroconidia size ranges different of *Fusarium oxysporum* f. sp. *phaseoli* isolates

Isolate	Microconidia (µm)	Macroconidia (3 septa) (µm)
*Fop*1	9x3.2	37x3.5
*Fop*2	9x3.6	42x3.5
*Fop*3	9x3.6	42x3.5
*Fop*4	8x3.0	35x2.9
*Fop*5	8x3.5	30x3.0
*Fop*6	10x3.4	41x3.5
*Fop*7	8x3.0	28x2.8
*Fop*8	9x3.5	40x3.4
Mean	9x3.4	37x3.3

3.2 Pathogenicity Variability

The pathogenicity of the *Fop* isolates obtained was assessed on a susceptible French bean variety "Amy". All of the 8 isolates under study were found to induce disease in French bean under study resulting in wilting, yellowing and death of plants (Fig. 4). However, the difference in pathogenicity between the isolates tested was significant ($P< 0.01$) (Table 3). The most pathogenic isolates were *Fop*3, *Fop*6 and *Fop*7 (Fig. 5). These isolates had a mean pathogenicity of 98.2, 91.9 and 90.7%, respectively. The least pathogenic isolate was *Fop*5 with a mean of 65.9% pathogenicity (Fig. 5). The un-inoculated control under similar conditions didn't show any *Fusarium* wilt symptoms. Differences in symptoms were distinct on foliage. At early stage, symptoms appeared as yellowing of the lower leaves and in later stages, drooping of the leaves was observed. In severely infected plants, lower leaves became chlorotic and eventually dried.

Fig. 4. French bean showing *Fusarium* wilt symptoms in pathogenicity test greenhouse experiments

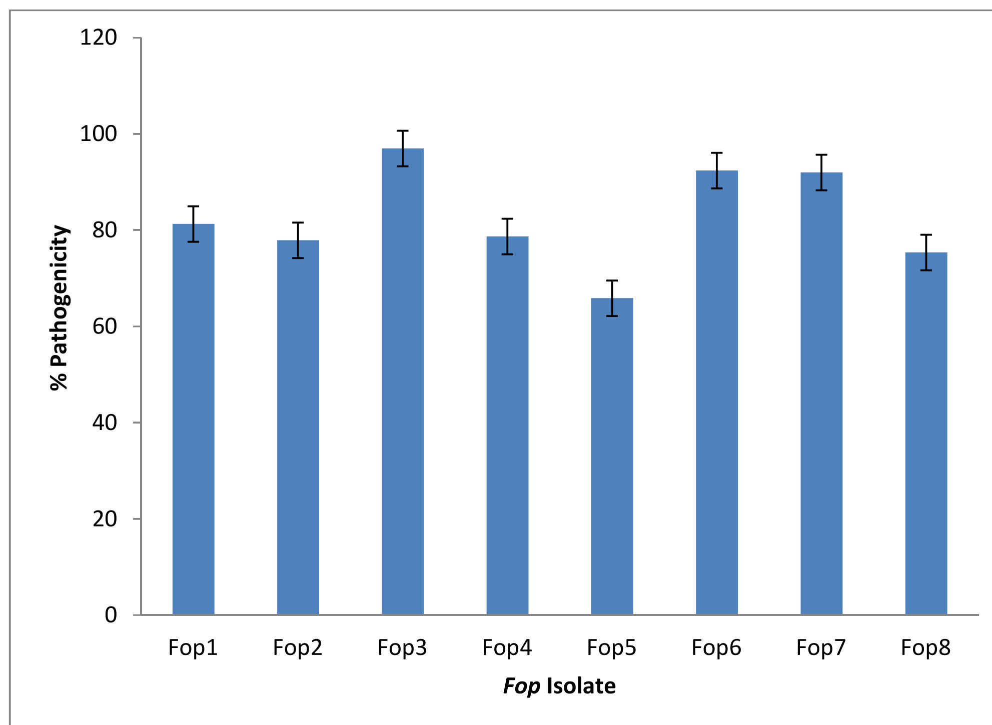

Fig. 5. Percentage pathogenicity of different *Fusarium oxysporum* f. sp. *phaseoli* on French bean (with standard error bars)

Table 3. Analysis of variance of pathogenicity of *Fusarium oxysporum* f. sp. *phaseoli* isolates on French bean

ANOVA						
Source of variation	**SS**	**df**	**MS**	**F**	**P-value**	**F crit**
Between groups	5325.163	7	760.7376	50.45754	5.52E-20	2.207436
Within groups	723.6857	48	15.07679			
Total	6048.849	55				

4. DISCUSSION

Considering that *F. oxysporum* f. sp. *phaseoli* is a serious pathogen of French beans, there was need to evaluate the differences in this fungus. This study has revealed that *F. oxysporum* f. sp. *phaseoli* isolates from the study region are diverse in terms of cultural, morphological and pathogenic characteristics. The isolates exhibited a high level of diversity in terms of cultural and morphological characteristics on PDA, SNA and CLA media. The isolates showed luxuriant, moderately luxuriant or scanty aerial mycelial growth. The growth rate on PDA also differed among the isolates with Fop8 having the highest growth rate. The mycelial texture was either fluffy or fibrous. The isolates growing on PDA were white, pink and purple. Most isolates had profuse sporulation. The isolates differed also in their macro- and microconidia sizes. In a previous study, Alves-Santos et al. [5] demonstrated the existence of variability in *Fop* isolates from Spain and Greece based on cultural and morphological characters. These results are also consistent with those reported by Mwang'ombe et al. [17]. They demonstrated a high cultural, morphological and pathogenic variability in *F. solani* f. sp. *phaseoli* attacking beans in Kenya. Suman and Mohan, [3] also demonstrated existence of cultural, morphological and pathogenic variability in *F. oxysporum* f.sp. *ciceri*. The differences obtained from this study could be traced to their in genetic make-up.

The results from this study further revealed that all the 8 *F. oxysporum* f. sp. *phaseoli* isolates were pathogenic to French bean. There was significant difference in pathogenicity between the isolates obtained ($P < 0.01$). The most pathogenic isolates were *Fop*3, *Fop*6 and *Fop*7 with mean pathogenicity of 98.2, 91.9 and 90.7%, respectively. The least pathogenic isolate was *Fop*5 with a mean of 65.9% pathogenicity. It was noted that none of the isolated prevented germination of seeds. The isolates showing very high virulence could depict an evolution based on adaptive mutation. The high prevalence of these aggressive isolates in the study area could be due to migration via irrigation water or planting materials.

The study confirmed that all the *Fop* isolates were pathogenic to the French bean. These results are inconsistent to those reported by Alves-Santos et al. [5] who indicated that *Fop* has many isolates that are nonpathogenic. However, the results of this study are consistent to those to those reported by Mwang'ombe et al. [17] where they demonstrated two categories of isolates based on virulence. However, in this study most isolates were highly virulent. A study by Francisco et al. [18] in Brazil also demonstrated a prevalence of higher difference in pathogenic potential among 25 isolates of *F. oxysporum* f. sp. *phaseoli.* Muriungi [19] also reported prevalence of highly pathogenic *Fop* strains in western region of Kenya. The results on cultural and pathogenicity characteristics are consistent with those obtained by Siddique et al. [20] in Bangladesh. In their study they reported that the colour of the *Fop* isolates varied from white to pink while the texture of the isolates was fluffy. Although they reported the highest pathogenicity at 97.22%, they concluded that the virulence of *Fop* isolates is highly variable.

Fusarium fungus reproduces mainly by asexual means. However, it may have parasexual cycle creating new genetic combinations that may account for the cultural, morphological and pathogenic differences among the isolates in this study. The parasexual cycle is depicted by three stages; anastomosis, heterokaryosis and karyogamy. A study by Silva et al. [21] confirmed molecularly the existence of different fingerprints of *F. oxysporum* isolates obtained from same geographical zone. The isolates clustered into different similarity groups. According to Fourie et al. [22] parasexuality arises by high mutations which may occur among the isolates. High Heterokaryosis has also been associated with continuous cultivation of the host crop [18]. The growth of the French bean in the study area is through continuous cropping. This is possible because of availability of water for irrigation from

a nearby permanent river. This practice may result to buildup of inoculum as the farmers tend to grow only one type of French bean variety. As a result, heterokaryosis may occur resulting into isolates with clear differences from the original population.

The high prevalence of pathogenic *Fop* isolates in the study area do not portend well for the economy of this area. There remains a serious threat to French bean farming in this region due to the high inoculum levels of *Fusarium* wilt pathogen. This pathogen cannot be controlled by use of chemicals. The existence of pathogenicity potential difference among the isolates obtained poses a threat to the use of resistant varieties. This information is relevant to crop breeders, farmers and plant pathologists.

5. CONCLUSION AND RECOMMENDATION

This study revealed cultural, morphological and pathogenic variability among the *F. oxysporum* f. sp. *phaseoli* isolates. Characterization of fungal pathogens is an important step in understanding development of strategies for their management. The cultural, morphological and pathogenic variability can be used for further development of local/region specific or even race specific resistant varieties of French bean and in developing disease control strategies. Future studies should focus on genetic characterization of *Fop* isolates from this region to give more insight on genetic diversity of the isolates. This will allow better conclusions to be made about *Fop* populations in the study area.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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