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MOLECULAR CHARACTERIZATION AND PATHOGENICITY OF *Fusarium falciforme* CAUSING WILT IN THREE IMPORTANT LEGUMES CROPS IN NIGERIA

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SUMMARY

Legumes, members of the Fabaceae family are susceptible to fungal pathogens causing wilts and root rots. This study aimed to examine the distribution and virulence of Fusarium wilt isolates on three legume crops: cowpea (*Vigna unguiculata* L.), soybean (*Glycine max* L.), and African yam bean (AYB, *Sphenostylis stenocarpa* H.). The investigation also sought to establish a relationship between *Fusarium* strains and their ability to infect various legume crops. Infected and withered roots were collected from three fields at the International Institute of Tropical Agriculture, Ibadan and isolated on Potato Dextrose Agar medium plates for identification. Pathogenicity test to show the relationship between *Fusarium falciforme* on the legume crops were investigated in a screen house experiment using completely randomized design (CRD). Each seedling was inoculated with 30 ml of *F. falciforme* suspension (5 x10^6 conidia ml). Data collected over 5 to 25 days after inoculation were analyzed using analysis of variance at 5% probability. All isolates were identified as *F. falciforme* through conventional and molecular characterization. To our knowledge this is the first report of *Fusarium* wilt in legumes caused by *F. falciforme* in Nigeria. The results indicated significant differences ($p \leq 0.05$) among the treatments applied to the crops, treatment effects were significant ($p \leq 0.05$) with AYB showing a notable difference compared to cowpea and soybean. This study showed that *F. falciforme* from AYB showed high level of virulence compared to strains from cowpea and soybean, and that AYB is resistance to *F. falciforme* strains from the other two crops.

Keywords: *Fusarium falciforme*, solarization, pathogenicity, virulence, resistance, seedling mortality

Legumes, belonging to the Fabaceae family, play a significant role in agriculture as valuable food and forage crops for high-quality meat and milk production (2;13). These versatile crops serve as stable food sources in various regions and are increasingly valued for their health benefits. Legumes are rich sources of essential nutrients, including plant protein, fiber ranging from about 20% to 30% of their dry weight, B-vitamins, iron, folate, calcium, potassium, phosphorus, and zinc, making them important components of a balanced diet (1). Despite their nutritional value and importance in agriculture, legume cultivation faces challenges in meeting yield expectations compared to other crops like cereals. However, their susceptibility to fungal pathogens causing wilts and root rots pose a significant challenge to legume cultivation. Plant diseases are recognized as one of the most

significant limiting factors in agriculture, with potential consequences ranging from yield reduction to complete devastation. The impact of diseases, including those caused by fungal pathogens, contributes to the relatively low and unstable yields observed in legumes (6). Among these diseases, *Fusarium* wilt stands out as a major soil-borne fungal infection that results in the blockage of water-conducting vessels (xylem) in plants, leading to wilting and often death. *Fusarium* species are commonly found in soil and are closely associated with plant roots, functioning either as parasites or saprophytes (10). Over the years, *Fusarium* species have been economically significant as plant pathogens, causing diseases such as crown rot, head blight, and scab in cereal grains and vascular wilts in various horticultural crops such as tomatoes, cucurbits, and bananas (8). Primarily, various host-specific forms of *Fusarium oxysporum* cause wilt. *Fusarium* wilts are caused by pathogenic strains of several species of *Fusarium*, including *F. eumartii, F. oxysporum, F. avenaceum, F. solani, F. sulphureum and F. tabacinum* (Plant Health Research and Diagnostics, 2007) which are usually very host specific. To attain sustainable and enhanced legume production, it is imperative to confront the challenges presented by fungal diseases affecting legumes and devise efficacious management strategies. Numerous studies have reported *Fusarium* wilt as the result of *Fusarium oxysporum* (9;11;13). Nevertheless, the connection between the pathogen, its impact on different crops, and cross-reactivity within each crop of study remains unclear. It is essential to gain knowledge and assess the characteristics of these pathogens through efficient screening techniques to establish effective control strategies. Therefore, the primary objective of this research is to enhance understanding of the distribution range and virulence capacity of each *Fusarium* isolate found in the legumes under investigation, with a focus on cross-reactivity.

MATERIAL AND METHODS

Sample collection

Infected roots with typical wilting symptoms of each crop (15 roots in total) were randomly collected from three different fields at International Institute of Tropical Agriculture (IITA), Ibadan Nigeria, West Africa. IITA is located at latitude 7.3^{0}N and longitude 3.45^{0}E in derived savanna agro-ecological zone of Nigeria.

Isolation of *Fusarium* sp. **from infected roots of each crop**

Infected roots from diseased plants of Soybean, Cowpea and African yam bean showing typical symptoms of root wilt were collected. The root samples were washed under running tap water to remove all visible soil; freshly infected tissue was selected for isolation. The infected tissue segment of about 5-10 mm^2 were excised from lesion margin on the root, disinfected with 10% sodium hypochlorite for two minutes. The tissue was rinsed in sterile distilled water (SDW) and blot- dried with sterile paper towel. The infected tissue segment was placed on Potato Dextrose Agar medium. **The plates were then** incubated after isolation at 26-28°C for 4 days at room temperature under 12 hrs of light and darkness.

Morphological and Molecular characterization of each isolate

Morphological characteristics

The plate was observed, and isolates intercepted were recorded. Purification of isolates were done by cutting small disc of each organism on the point of isolation with the aid of scalpel blade with handle and

inoculated on a freshly PDA medium plate. The plates were then incubated after isolation at 26-28°C for 4 days at room temperature under 12hrs of light and darkness. A pure culture was obtained and stored in the refrigerator at 4°C for further use. Growth characteristics on PDA and microscopic examination of size, shape, and number of septate of the macro conidia, micro conidia, and presence of chlamydospores were used to determine the morphological characteristics according to identification manual by Paul *et al*.,1983.

DNA extraction

All *Fusarium* isolates were cultured on PDA medium and incubated for 5 days. One hundred milligram of fungal mycelium was taken into sterile mortal and macerated in 1ml of DNA extraction buffer containing proteinase K (0.05 mg/ml). The extract was transferred into 1.5 ml Eppendorf tube and 50 µl of 20% Sodium Dodecyl Sulphate (SDS) was added to the extract, incubated in a water bath at 65°C for 30 minutes (for lysis to take place). The tubes were allowed to cool to room temperature and 100 µl of 7.5 M potassium acetate was added and mixed briefly. The extract was centrifuged at 13000 rpm for 10 minutes and the supernatant was transferred into 1.5 ml sterile tubes. Two-third volume of cold isopropanol was added to the supernatant and the tubes were gently inverted 3-5 times and incubated at -20°C for 1 hour. The sample was centrifuged at 13000 rpm for 10 minutes and supernatant was discarded. An aliquot of 500µl of 70% ethanol was added to the precipitate and centrifuged for 5 minutes at 13000 rpm, the supernatant was carefully discarded to retain the DNA pellet. Ethanol was eliminated through a meticulous decantation process and the DNA pellets dried in incubator at 37 °C for 10-15 minutes and re-suspended in 50 µl of sterile distilled water. The DNA was aliquot (small portion) and stored at -20 °C for further laboratory analysis.

PCR analysis

The universal primer pair, ITS1 and ITS4 were used in the PCR reactions as described by White *et al*. (1990). The primers amplified the ITS region of the rRNA operon. The amplification of the fragments was performed using thermal cycler (Biometra®TProfessional). The reaction mixture contained 2.5 µL of reaction buffer, 1.5 mM $MgCl_2$, 0.3 U of Taq DNA polymerase, 0.2 mM dNTPs mix, 0.2 µM of each ITS primers (IDT, Belgium) and 2 µL of diluted 1:50 (v/v) (100 ng/µL) total nucleic acid extract. Oligonucleotides used for nucleic acid amplification were ITS-1: GGCACACATGCAAATGAATGC and ITS-4: CACCAGTAGAGTGAACATAG. The PCR conditions include a cycle of initial denaturation at 94°C for 5 min, followed by 35 cycles of each cycle comprised of 30secs denaturation at 94°C, 30secs annealing of primer at 55°C, 1.5 min extension at 72°C and a final extension for 7min at 72°C (14)

Gel electrophoresis

To quantify the PCR product, gel electrophoresis was conducted using 2% final concentration of agarose gel using 120V for 1 hour. The gel was stained with Fluorosafe dye (1st Base Company, First Base Laboratories Sdn. Bhd. Seri Kembangan, Selangor, Malaysia). A 100bp ladder was used as the marker of the fragments size. The gel was captured with ultra violet (UV) radiation using lab image doc (BioRad®).

Nucleotide sequence and phylogenetic analysis

The PCR products were purified and sequenced at the IITA Bioscience Unit, Ibadan. The purified PCR products were sequenced using ABI3730XL sequencer. **BioEdit** sequence alignment editor at

https://bioedit.software.informer.com/7.2/ was used for the sequence alignment edit and the sequences were analyzed and compared to the database from National Center of Biotechnology Information (NCBI) at https://www.ncbi.nlm.nih.gov for Basic Local Alignment Search Tool (BLAST). The sequences were aligned for phylogenetic analysis using Clustal W in Molecular Evolutionary Genetics Analysis version X (MEGA X) (14). The Maximum Likelihood analysis was done by using Tamura-Nei model generated a phylogenetic relationship from aligned sequences for each data set. About 1000 replications of bootstrap analyses supported the phylogenetic groupings.

Pathogenicity test

Pathogenicity of *Fusarium falciforme* was tested on three varieties of cowpea (IT14k-2029, IT08k-150-12, 14k-2022), soybean (TGx 1485-1D, TGx 1880-3E, TGx 2009-16F) and African yam bean (Tss 96, 296, 59),. Three seeds of each crop were sown in 10 cm diameter pots and allowed to grow for two weeks and later thinned to two plants per pot. Inoculum consisted of spore suspension obtained from 14 days old culture on PDA was used and each seedling was inoculated by pouring 30 ml of the suspension (5 x10^6 spores per ml quantified through haemacytometer) to the upper part of the soil which serve as the base of the plant. Each treatment consisted of six seedlings (replicates). The experiment was arranged in a Completely Randomized Design (CRD). Symptoms development were observed every five days for 5weeks. Disease incidence (DI) was calculated based on the root associated symptoms with modification (3). Plants were considered infected when they expressed symptoms-yellowing of lower leaves, wilting of leaves, defoliation or marginal necrosis of the remaining leaves. The five treatments evaluated are listed as follows:

1. Each plant was inoculated with *Fusarium falciforme* isolated from each host crop (Single)
2. *Fusarium falciforme* isolated from each crop cross-reacted with each other (Cross inoculation on first crop)
3. *Fusarium falciforme* isolated from each crop cross-reacted with each other (Cross inoculation on second crop)
4. Mixture of the three *Fusarium falciforme* was inoculated with on the crops cowpea, soybean and African yam bean (mixed inoculation)
5. Each plant supplemented with distilled water served as control

The treatments exhibited statistically significant differences concerning the type of crop and the readings taken at 5, 10, 15, 20, and 25 days after inoculation (DAI). Data were systematically collected for analysis. Mean values were calculated for each treatment and crop, accompanied by 95.0% confidence intervals for these means at each respective day.

RESULTS

Fusarium falciforme, along with other related organisms, were isolated from the 15 samples collected from the three different crops of study. Among the five organisms (*Collectotrichum gloeosporioides, Botryodiplodia theobromae*, *Cladosporium spp*, *Fusarium falciforme*, and *Fusarium verticillioides*) isolated, *F. falciforme* exhibited the highest frequency on the agar plate. Observations on the plates revealed *F. falciforme as the* dominant organism among its associates. The percentage incidence of *F. falciforme* was notably high (86.7%), indicating its

significance and the dominant causal organism among others (Table 1).

The mycelial growth pattern, colony color, and pigmentation of the isolate (*F. falciforme)* were examined using pure cultures grown on PDA medium. The aerial mycelia exhibited a white ring fluffy growth with a light purple center for the soybean isolate. Similarly, the African yambean isolate displayed a white ring fluffy growth, while the cowpea isolate show cased white fluffy growth with concentric rings. On the reverse side or abaxial surface of each isolate, pigmentation ranged from dark orange to light orange. Microscopic analysis of the isolates revealed the presence of microconidia, macroconidia, chlamydospores, and sporodochia (as seen in Plate 1 and 2).

The radial growth of each isolate when cultured on PDA medium was assessed, it was noted that within the initial 2 days, *F. falciforme* exhibited the fastest mycelial growth on African yambean, while on cowpea, it displayed the most rapid and extensive mycelial growth and sporulation at 14-day. A comparison of the three isolates from the different crops revealed that *F. falciforme* on soybean exhibited the slowest growth at third day of incubation. Consequently, among the three isolates, *F. falciforme* on cowpea demonstrated the highest percentage growth on days 3, 6, and 9 (as shown in Figure 1).

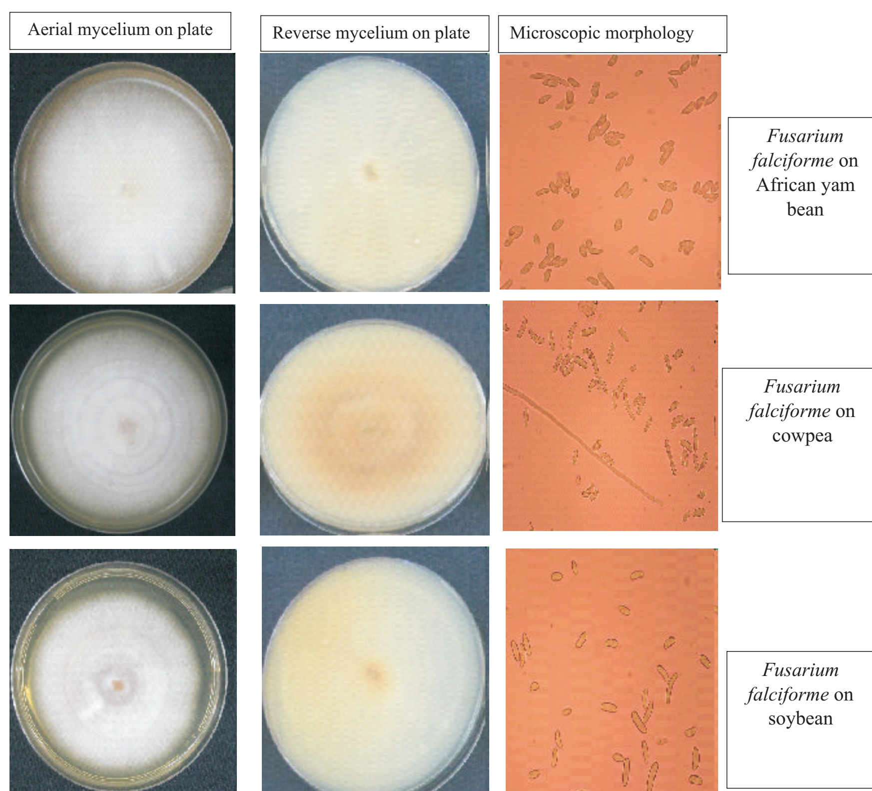

Plate 1: Morphological characteristics of each isolate on PDA plate

Table 1: Percentage occurrence of fungi isolated fromwilted Soybean, African yam bean andCowpea

Crop	Percentage (%) incidence of Pathogen intercepted				
	Fusarium falciforme	***Lasiodiplodia theobromae***	***Colletotrichum gloeosporioides***	***Cladosporium* spp.**	***Fusarium verticillioides***
African yam bean	66.7	13.3	13.3	6.7	0
Cowpea	86.7	0	0	6.6	6.7
Soybean	73.3	26.7	0	0	0

Table 2: Morphological characteristics of the three isolate of *F. falciforme* (as shown in plate 1)

Crop	Color of aerial mycelium [a]	Pigmentation [b]
African yam bean	Fluffy, ring growth and whitish	Light orange
Cowpea	Fluffy, concentric ring growth and white	dark orange
Soybean	Fluffy, ring growth with light purple at the center, and whitish	Light orange

[a] Colony colour were determined by observing the upper surface of the colony.
[b] Pigmentation was determined by observing the lower surface or abaxial of the colony.

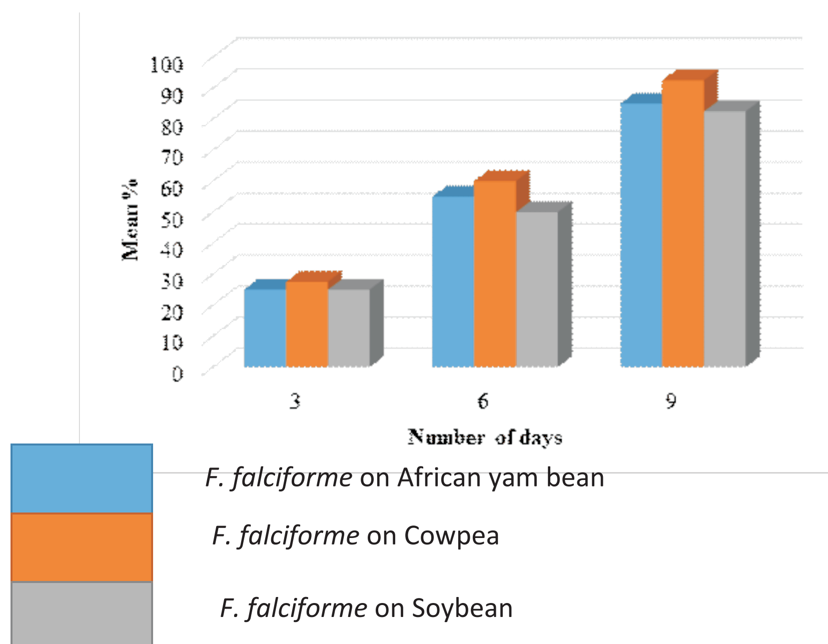

Fig. 1: Mean percentage of the growth rate at 3, 6 and 9 days

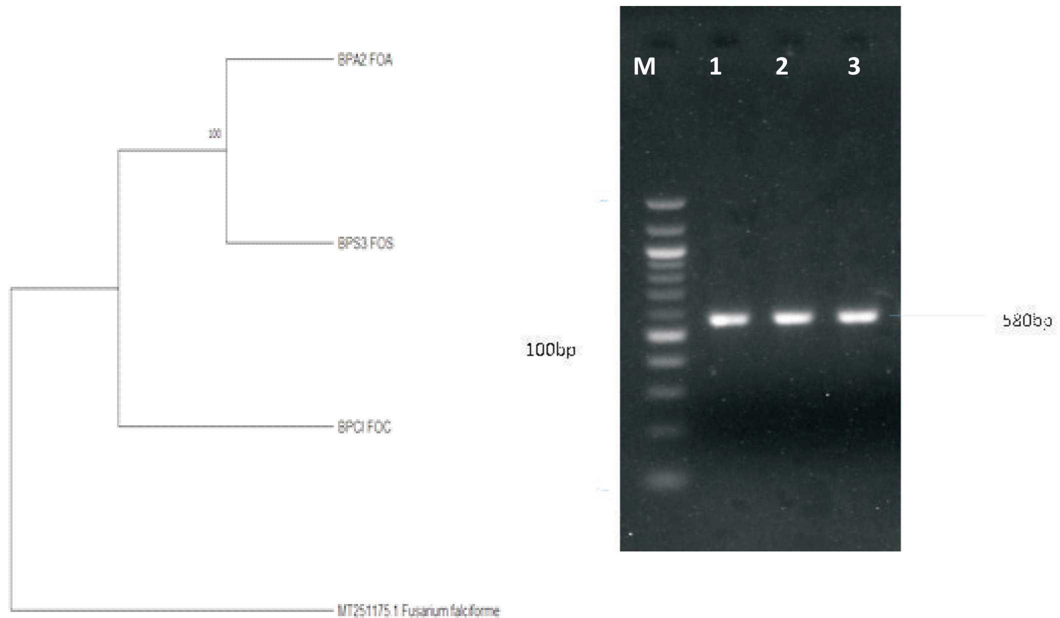

Plate 2: Gel picture obtained from the DNA and Phylogenetic tree of the three isolates

Effect of treatment on the crop

At 5DAI, the distinctions in treatments' effects on crops were evident, as highlighted in Tables 2. A significant difference was found between African yam bean and cowpea, as well as between cowpea and soybean. However, there was no statistically significant between the effects on African yam bean and soybean. Contrarily, cowpea deviated from this pattern, exhibiting notable differences.

At 25 DAI,, Table 3 showed the means for each crop and treatment. . Significantly different outcomes were noted between African yam bean and cowpea, as well as between African yam bean and soybean. Conversely, no significant difference was detected between cowpea and soybean. When all days and treatments were examined collectively, Figure 4 illustrated the mean disparities among the various crops.

Table 3: ANOVA on the severity of *Fusarium* wilt on African yam bean, cowpea and Soybean for at 5 DAI

Source	*Sum of Squares*	*Df*	*Mean Square*	*F-Ratio*	*P-Value*
MAIN EFFECTS					
A: Crop	3.12593	2	1.56296	8.07	0.0005*
B: Treatment	2.26667	4	0.566667	2.92	0.0236*
RESIDUAL	24.8	128	0.19375		
TOTAL (CORRECTED)	30.1926	134			

* denotes a statistically significant difference at ($P < 0.05$) based on

Table 4: ANOVA on the severity of *Fusarium* wilt on African yam bean, cowpea and Soybean for 25 DAI

Source	*Sum of Squares*	*Df*	*Mean Square*	*F-Ratio*	*P-Value*
MAIN EFFECTS					
A: Crop	40.1926	2	20.0963	26.24	0.0000*
B: Treatment	199.037	4	49.7593	64.97	0.0000*
RESIDUAL	98.0296	128	0.7659		
TOTAL (CORRECTED)	337.259	134			

* denotes a statistically significant difference at (P < 0.05) based on LSD's test

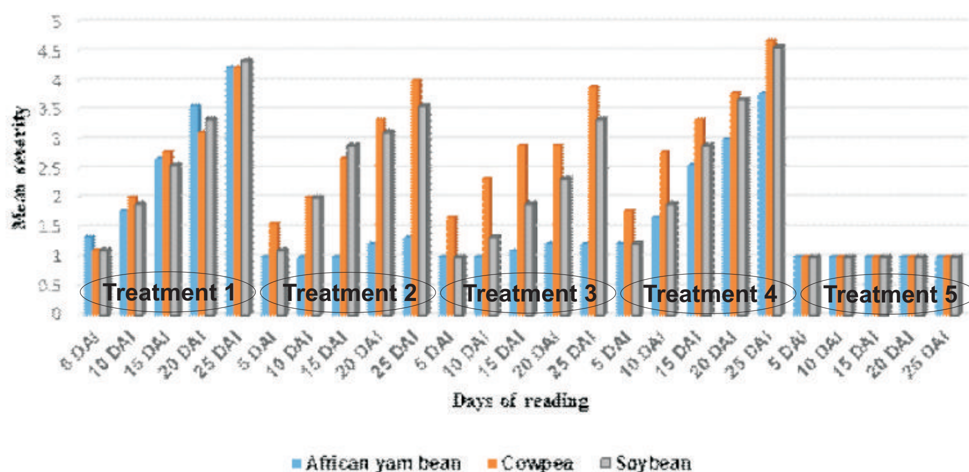

Fig 4: Mean of each treatment after inoculation

DISCUSSION

The findings of this study contribute valuable insights to the understanding of *Fusarium falciforme* isolates in legume crops in Nigeria. While the study establishes that isolates from African yam bean, soybean, and cowpea share the same Genus and species (*F. falciforme*) based on conventional identification methods, it emphasizes the novel aspect of molecular characterization, marking the pioneering report of such a study in Nigeria.

The work aligns with previous research on *Fusarium* species and their impact on crops. The conventional identification method based on morphological characteristics and microscopic traits corresponds to established practices in fungal taxonomy, this is similar to the work done by Dongzhen Fang *et al* (5). The molecular characterization aspect of the study reflects the increasing use of molecular techniques for precise identification and classification of fungal species, enhancing the accuracy of taxonomic which is consistence with previous of study on confirmation of pathogen identity with molecular characterization (7).

The study's pathogenicity tests revealing variations in virulence levels among isolates from different crops build upon earlier research investigating the pathogenic potential of Fusarium species in various host plants which was in line with the work done by (16). The observation that *F. falciforme* from African yam bean exhibits heightened virulence compared to isolates from soybean and cowpea introduces a new dimension to understanding the host-pathogen interaction within the Fusarium genus.

The study's recommendation to use non-host crops like African yam bean as a control measure to echoes the principles of integrated pest management, emphasizing the importance of crop rotation and utilizing non-susceptible plants to manage soilborne pathogens which is line with benefit of crop rotation as mentioned Neher *et al* (2019). The idea that planting a non-susceptible crop like African yam bean can reduce the inoculum level of *Fusarium falciforme* and alleviate the need for chemical treatments aligns with sustainable agriculture practices.

In summary, this study builds upon existing knowledge by combining conventional and molecular identification methods, highlighting variations in virulence levels among *Fusarium falciforme* isolates from different legume crops, and proposing a practical and sustainable control measure through the use of non-susceptible crops. The recommendations align with established practices in fungal taxonomy, molecular characterization, and integrated pest management.

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