Identity of Fusarium species associated with collar rot and wilt in passion fruit (Passiflora edulis)

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Abstract

Background:

Despite the immense contribution of passion fruits to people's livelihood on a global scale, the crop's productivity remains low owing to fungal diseases causing up to 100% loss. Fungi are highly variable and the identity of species or variates responsible for recently devastating passion fruit wilt and collar rot diseases had not been characterized. This study was aimed at identifying pathogens causing wilt and collar rot symptoms in passion fruits.

Methodology:

Fungi were isolated from diseased samples collected from three locations in Central Uganda to identify *Fusarium spp* associated with collar rot and wilting of passion fruit. This was established by differentiating mycelium pigmentation on Potato Dextrose Agar (PDA), examining slides at X40 magnification under a light microscope for specific macro and microconidia, and amplification with specific Transcription Elongation Factor-1α, TEF 1α primers for identification of *Fusarium spp.*

Results:

It was revealed that wilting was associated with a single species, out of 6 selected isolates from the suspected wilted plant, 3 were *Fusarium spp* associated with the disease in the field but only one of these isolates was proved to be a pathogenic type *Fusarium oxysporium.* Collar rot was associated with one pathogenic *Fusarium spp* out of the 6 selected isolates.

Conclusion:

The results indicate that collar rot and *Fusarium* wilt are each caused by specific strains of *Fusarium* pathogens.

Recommendation:

The identification of pathogenic *Fusarium* in farmers' orchards is a starting point for designing effective disease management measures against the predominant fungal pathogenic variants in passion fruits.

Keywords: Mean disease index, pathogenic fungi, passion fruit orchard, polymerase chain reaction, relative disease damage, Submitted: 18th/09/2022 Accepted: 12th/10/2022

1. Introduction

Passion fruit is a member of the genus *Passiflora*, which is a high-value horticultural crop

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world The crop originated in Southern Brazil but was widely distributed in the tropics and subtropics during the 19th Century (Amata, 2009). Brazil is the world's largest producer of passion fruit, with an estimated growth area of 44,300 ha and a fruit yield of 615, 196 tones (FNP 2009). Other countries growing commercial passion fruits include South Africa, Kenya, Zimbabwe, India, Israel, USA, Peru, Ecuador, Columbia, Venezuela, Trinidad and Tobago, Australia, Hawai, Netherlands, Newzealand, and Uganda. Production is, however, constrained by various factors mainly diseases.

There are several diseases caused by *Fusarium* in passion fruit, these include; collar rot caused by *F. solani*, wilt by *F. oxysporum* and die back by *F. solani*, *F. subglutinans*, *F. semitectum*, *and F. pseudoanthophilum* (Amata, 2009). Globally, diseases caused by soil-borne pathogens including *Fusarium*s, are economically important as they reduce useful life, with orchids living to be renewed every 2 years or less (Alabauvette, 1999). Among these diseases stands out the fusarious passion fruit wilt caused by *Fusarium oxysporum. f.*sp *Passiflorae* (Fop) (Amata, 2009). Fop occurs in several passion fruit-producing regions of Brazil and affects crop yield (Alabauvette, 1999). Two fungal diseases of passion fruit namely *Fusarium* wilt and collar rot,

are economically important in most of the crop's growing areas including Eastern Africa (Emechebe and Makumbi, 1976). The diseases are widespread and limit the longevity of established orchards to between 2 to 5 years, and they cause yield losses in the range of 40 to 100% (Wangungu, 2010).

Fop is both seed and soil-borne. It produces resting spores called chlamydospores for survival in the soil in absence of a host plant. The conidia morphology identification includes; the structure of top and foot cells, the nature of aerial mycelium, pigmentation/spore color, and the presence of chlamydospores, sporodochia, and promates. The growth rate of Potato Dextrose Agar is used for identification, as is the size, length/width ratio, shape, and septation of macroconidia. All can be done concerning The *Fusarium* Laboratory Manual (Leslie, 2006). Fs survive for years as chlamydospores in the soil and may be spread by any practice resulting from the movement of infected soils. Infected seedlings are also responsible for spreading the pathogen (Fischer, 2008).

As in the case of Fop, conidia morphology is used for identification concerning The *Fusarium* Laboratory Manual (Leslie, 2006). Morphological, cultural, and pathological characterization of the fungal pathogens associated with wilting and collar rot in passion fruit offers an indication of the genetic diversity of these fungi and supports efficient management of the diseases through the host resistance approach. This would subsequently reduce major losses in plant yield and quality. The diversity of pathogenic *Fusarium* in farmers' orchards needs to be determined so that effective control measures against predominant pathogenic variants can be

This is a precondition for the efficient management of diseases and the development of host resistance in passion fruit cultivars (Ssekyewa, 1999). The range of diversity in pathogenic *Fusarium*s associated with wilt and collar rot from farmers' orchards is had not been validated. In this study, we, therefore, sought to understand the diversity of *Fusarium* species that devastate passion fruit orchards.

2. Materials and methods

2.1. Microorganism cultivation and conidia observation

Sampling was carried out in Mpigi, Mukono, and Wakiso Districts, which are popular for passion fruit growing in Uganda. Orchards were inspected for plants exhibiting Fusarium wilt and collar rot symptoms and samples including vine, roots, and soil were collected. The collected samples were transported to the National Agricultural Research Laboratories (NARL), Kawanda for further laboratory analysis. Laboratory samples were surface sterilized with 3.85% m/v sodium hypochlorite for 15min and 70% ethyl alcohol for 10 min or with 96% ethanol for 5 min and 10% silver nitrate for 15 min. Aseptically cut twig

pieces of approximately 1-2mm length were routinely cultured on potato dextrose agar (PDA) (Eur Pharm, Laboratories Conda S.A) which was prepared with 11.5g ml-^{1} powder water, supplemented with ampicillin (A01040.0010; Duchefa Biochem Netherlands) at 200μg per ml. Cultures were then incubated at 28°C and observed every 24 hrs over at least 3 days, to observe established fungal mycelium on PDA and subcultured on PDA continuously. Fusarium selection and purification were done using Fusarium selective medium PCNB (Pentachloronbenzene) composed of peptone 15g, potassium dihydrogen orthophosphate 1g, magnesium sulphate hydrate 0.5g, pentachloronitrobenzene 1g, Agar 20g l-1 water.

Spore suspensions were diluted 1/100 with distilled water and 10μl pipetted onto a microscope slide. A coverslip was carefully placed on top to avoid air bubbles and the slide was examined at X40 magnification under a light microscope. Spore counting was done using a microscope with the aid of a haemocytometer (1/10mm deep, bright line; Boeco, Germany), which was carefully cleaned with tissue paper on all surfaces to ensure dryness. Approximately 10μl of spore suspension was loaded onto the counting chambers. Coverslip was placed onto the sample and viewing was done using an optic microscope (Orthoplan, Germany) at 40X following the manufacturer's instructions.

2.2. DNA extraction from Microorganism

Sample material collected from the 3 districts was used for DNA extraction. Sterile sea sand

was used in DNA extraction from mycelia fungal colonies to aid maceration of mycelia as well

as cause cell lyses. 200μl of TES extraction buffer (0.2 M Tris-HCL pH 8, 10mM EDTA pH8, 0.5M NaCl, 1% SDS) was added to the sample in a motor. Macerated tissue suspension was transferred to 1.5mm microcentrifuge tubes. Samples were then vortexed for 30 sec and then placed in a water bath at 65°C for 30 min. One-half volume (250μl) of 7.5M Ammonium acetate was added to the samples which were then mixed and incubated in a refrigerator for 10 min, then centrifuged (tabletop centrifuge 5415D; Eppendorf) at 13,200rpm for 15 min, supernatant was then transferred to a new tube. To 500μl of the sample, 500μl of ice-cold isopropanol was added and the sample centrifuged for 10 minutes at 13,200rpm. Supernatant was decanted and each DNA pellet was washed with 800μl of cold 70% ethanol and centrifuged for 2 min at 13,200rpm. Pellets were air dried on clean sterile paper towels for 40 min. 50μl of RNASE free water was added to resuspend the pellets, which were then incubated at -20°C for 30 min. The quality of extracted DNA was then assessed by electrophoresis on an Agarose gel (0.6g of agarose powder and mixing in 50ml of 1X TAE buffer). In electrophoresis, 1.5μl of DNA loading dye was mixed with 5μl of the RNASE free water and 5μl DNA, these were thoroughly mixed and loaded into wells of the gel (5.5μl of the sample was loaded in each well). The gel was then run in the electrophoretic tank for 50 minutes and stained with ethidium bromide for 10 min. Bands on the gel were documented using a gel documentation system (Gene Snap Product version 7:09; Syngene).

2.3. Identification of the various Fusarium sp

PCR was performed using primers (Table 1) developed for specific and rapid detection of

Fusarium sp. and F. solani i.e. TEF-Fu 3 and TEF-Fs 4 respectively (Matsubara et al., 2004).

2.4. Evaluating fungal pathogenicity

Pathogenicity testing of isolated fungi was carried out on potted test plants under insect proof screen-house conditions. Seeds of susceptible (Passiflora edulis) and resistant (Kawanda hybrid) were planted in each of these pots for Fusarium pathogenicity assays. Other plant host species evaluated for Fusarium sensitivity included Solanum lycopersicum (tomatoes) and Solanum anguivi. Lam (African eggplant "katunkuma" Luganda). For each isolate collected, tests were carried out on host plant seedlings planted in triplicates per pot with 2-3 pot replicas and fertilized every 2 weeks. Inoculation of test plants was done using conidia harvested by adding sterile water to 14-day-old

Table 1: **1:** List of Primers developed for rapid detection of Fusarium sp. and F. solani

Prime code	Primer sequence	Gene	Amplified product size	Specificity	PCR programme
TEF-Fs4f	ATCGGCCACGTC-GACTCT	TEF-1α	658bp	F. solani Fig.15	40 cycle; 94°C for 1min, 58°C for 1min, and72°C for 2 min
TEF-Fs4r	GGCGTCTGTTGATTGT-TAGC				
TEF-Fu3f	GGTATCGACAAGCGAAC-CAT	TEF-1α	420bp	Fusarium sp. Fig.15	40 cycle; 94°C for 1min, 58°C for 1min, and72°C for 2 min
TEF-Fu3r	TAGTAGCGGGGAGTCTC-GAA				

cultures grown on PDA at 25o C and scraping the surface of the culture with a sterile glass slide. The resulting conidial suspension was filtered through two layers of cheesecloth to remove mycelia fragments. The concentration of the spore suspension foruse in inoculation was determined by using a haemocytometer (1/10mm deep, bright line; Boeco, Germany) under optic microscopy (Orthoplan, Germany) at 40X magnification. Theconcentration of spores was adjusted to the appropriate working inoculums dilution and adjusted to 1 x 106 conidia per ml. Plant host seedlings were inoculated at the six-leaf stage (Altnolk, 2005) with 5mls of the conidial suspension (Yoshiteru et al., 1996; Romberg and Davis, 2007). Re-infections were alternatively carried out with agar cubes of grown fungal mycelium. Foliar symptoms of each test plant were scored once every week after inoculation utilizing a scale of 1-5 (where: 1 = no symptoms; 2 = slight wilt and yellowing of the lowest leaves; 3 = half of the leaves wilted or showing yellowing; 4 = almost all the leaves wilted or showing yellowing; and 5 = all the leaves wilted, showing yellowing or plant died) (Mwaniki, 2011).

2.5. Ranking of disease scores

Inoculated plants were monitored daily for wilt development. The type of symptoms and extent of disease severity were recorded. To confirm the infectivity of the isolates, infected shoot tissues were randomly cultured on PDA. Cultural and morphological characteristics of Fusarium spp. colonies were recorded.

The response for each genotype and cultivar were determined against a mean disease index

3. Based on the following formula (Matsubara et al , 2004).

$$MDI = \Sigma\left(\frac{Number\ of\ diseased\ plants\ x\ degree\ of\ symptoms}{Total\ number\ of\ plants}\right)$$

Key interpretation for MDI: 0-1, least virulent; 1.1-2, moderately virulent; 2.1-3, virulent; 3.1-4, most virulent

Relative percentage damage was also established from the MDI based on the following formula;

$$Relative\ \%\ damage = \left(\frac{Sample\ MDI - control\ MDI}{Control\ MDI}\right) x\ 100$$

3.1. Statistical analysis

Disease index data was subjected to ANOVA and establishment of least significant differences of means at 5% error margin using GenStat 12th edition.

4. Results:

4.1. Morphological characteristics of various fungi

Pure isolations from diseased samples in central Uganda showed mainly two-color variations on PDA, mainly orange/cream and purple/violet mycelium from the sample colonies. Exceptionally, one sample from Mpigi 1 and one from Mukono 2 showed white and black pigmentation respectively (Figure 1, picture 5 and 4). Wakiso 1 district; 1 sample from Kawanda, had orange/cream mycelium (Figure 1, picture 1) and one from another farm in Entebbe in the same district was purple/violet (Figure 1, picture 6). Mukono district; the mycelium from one of the isolates X was purple/violet (Figure 1, Pictures 2 and 3) and another was black (Figure 1, picture 4). Mpigi District; mycelium colony on PDA for fungal isolate X from one of the two farms was white (Figure 1, picture 5) while another isolate X from the same district was purple/violet (Figure 1, picture 7) various fungi were isolated from the different districts according to Table 2.

Figure 2A shows an orange/cream fungus isolated from Kawanda (Wakiso) and re-isolated from diseased passion fruits. Macroconidia are morphologically wide, and straight, apical morphology was blunt or rounded, and basal morphology had a distinct foot shape, straight to almost cylindrical, usually with a notch or a rounded end. The number of septa was usually from 7. Microconidia; are oval with 0 or 1 septa.

In Figure 2B, purple fungus X from Mukono. In Figure 2C, fungus X from Mpigi (all purple/violet fungal isolates, mgX 40). Macroconidia were morphologically short to medium length, relatively slender, and thin-walled. The apical morphology was tapered and curved, sometimes with a slight hook (Figure 2C). Basal cell morphology foot shaped to be pointed; the number of septa is usually 3. Microconidia were oval or kidney-shaped and usually with no septa. Other black and white fungi were discarded since they could not colonize PCNB and had no visible Fusarium-like spores.

A, cream pigmented fungi from Wakiso; B and C, purple-pigmented fungi isolated from collected from Mukono, Mpigi, and Wakiso. They all had Fusarium like spores (macro and microconidia) as documented in the Fusarium Laboratory Manual.

4.2. Identification of various Fusarium spp

Identification of Fusarium sp. and F. solani for the Transcription Elongation Factor-1α was done and PCR results using primers TEF-Fs 4 and TEF-Fu 3 designed for specific detection of F. solani and Fusarium sp. respectively are shown below. Details of primer conditions are elaborated by Arif et al. (2012).

Figure 3 shows cream re-isolate from farm 6 (1), purple isolate from Mukono (2), purple isolate from Mpigi (3), purple isolate from Wakiso (4), Foc (positive control Fusarium oxysporium f. sp cubensi (5)) and purple re-isolate from P. edulis (6) from Mpigi same as 3. Primers TEF-FS4 specific to F. solani amplified DNA from isolate 1 from Kawanda giving a 658bp product confirming isolate 1 to be Fusarium solani. Primer TEF-Fu3 for general detection of Fusarium spp amplified DNA from four isolates giving a 420bp product. This clearly shows that others 2, 3, 4, 5, and 6 are other Fusarium species other than F. solani.

Lanes are Marker (M), Cream re-isolate from Kawanda (1), Purple isolate from Mukono (2), Purple isolate from Mpigi (3), Purple isolate from Wakiso (4), Foc (positive control, Fusarium oxysporium f.sp cubensi) (5), and Purple re-isolate from P.edulis from Mpigi (6) same as (3).

4.2.1. Values for the selected various fungi

Results show there is a significant difference among isolates for disease indices with $p<0.001$ and relative damage. Both the mean disease index (MDI) and relative % damage show that cream fungus from farm 6 (Wakiso 1) was the most destructive with percentage damage of 58.8% (Table 2), followed by purple fungus from farm 5 (Mpigi 2) with 29.4% and then purple fungus from farm 4 (Wakiso 2) with 17.6%. Some fungi resulted in lower disease levels, thus

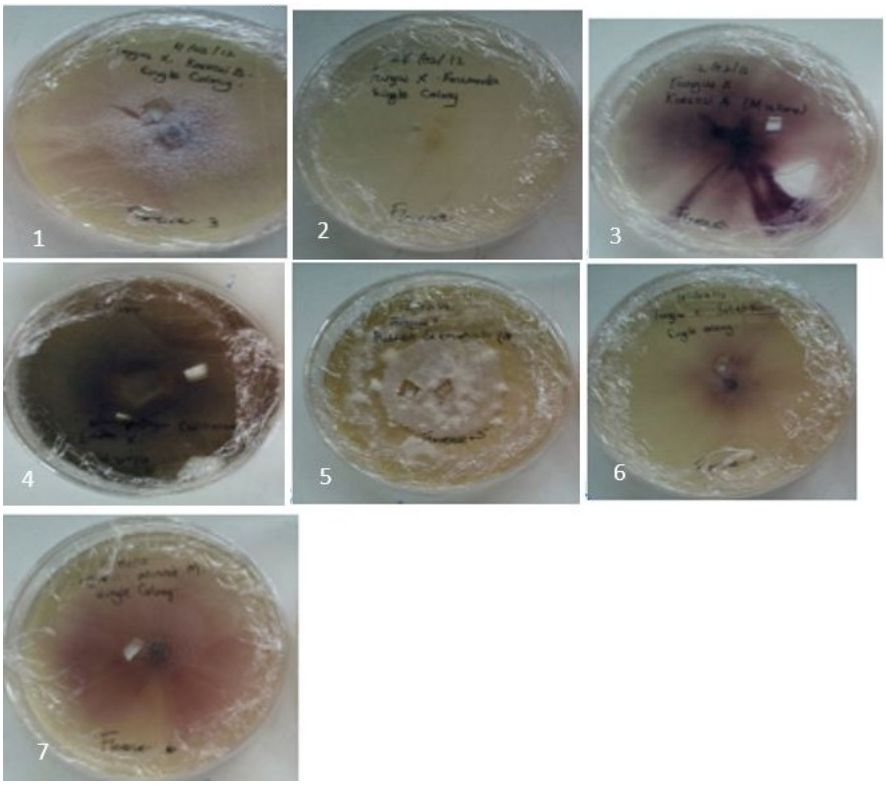

Figure 1: Mycelium characteristics of fungus X on $\frac{1}{4}$ PDA from the various sampled districts 1, cream/orange; 2, purple/violet; 3,purple/violet; 4, black; 5, white; 6, purple/violet; 7 purple/violet

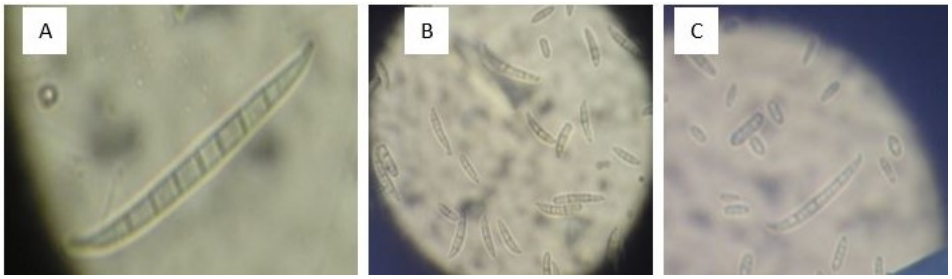

Figure 2: Mycelium characteristics of fungus X on $\frac{1}{4}$ PDA from the various sampled districts. A, cream pigmented fungi from Wakiso; Band C, purple pigmented fungi isolated from collected from Mukono, Mpigi andWakiso. They all had Fusarium likespores (macro and micro conidia) as documented in the Fusarium Laboratory Manual.

negative percentages. Examples of such were farm 1 (Mukono 1A), farm 1 (Mukono 1B), farm 2 (Mukono 2), and farm 3 (Mpigi 1) reduced damage in terms of leaf discoloration, defoliation, and general plant appearance as compared to the control.

5. Discussion:

The microbial cultures revealed that most of the *Fusarium* species were the purple/ violet pigmented and these were from Mukono. Mpigi and Wakiso. The least common had cream colonies and were isolated from Wakiso. One isolate from Kawanda, Wakiso district was identified as *F. solani* and 3 other purple isolates were identified as *Fusarium* using the TEF-Fs 4 and TEF-Fu 3 primers respectively. One cream fungus from Kawanda and one Purple/violet fungus from Mpigi were proved to be pathogenic to passion fruit causing collar rot and *Fusarium* wilt respectively.

The commonest *Fusarium* sp according to

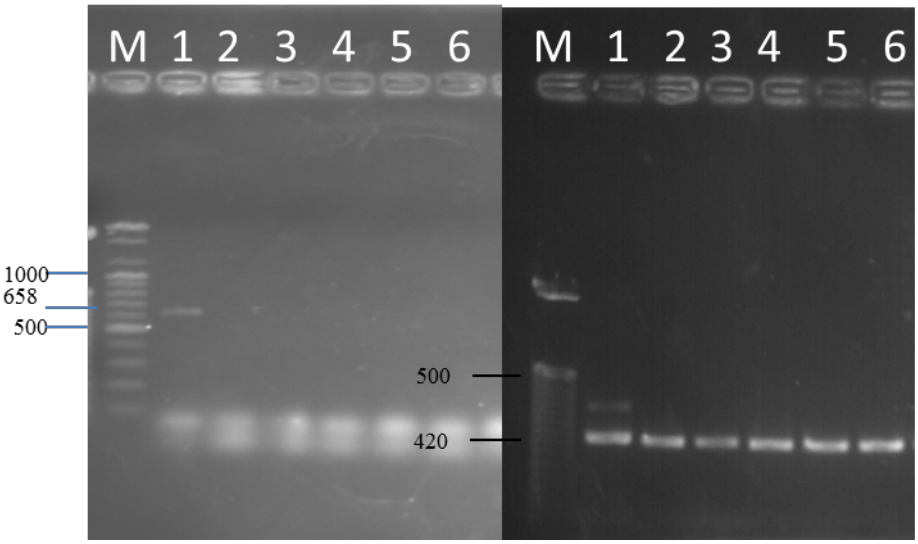

Figure 3: PCR product amplification of fungal samples with primers TEF-Fs4 658bp (A) and TEF-Fu3 420bp (B)

Table 2: Calculations of mean disease index (MDI) and relative damage (RPD) for P. edulis

Isolate	**MDI**	**RPD (%)**
Wakiso 1	3.271a	58.8
Mpigi 2	2.274b	29.4
Wakiso 2	2.202bc	17.6
Control	1.867cd	0.0
Mukono 2	1.788de	-5.9
Mpigi 1	1.725de	-17.6
Mukono 1	1.663de	-5.9
Mukono 3	1.459e	-17.6
l.s.d (α=0.05)	0.2182	

Values followed by the same letter are not significantly different.

colour and conidial characteristics were the purple/violet isolate from districts of Mukono, Mpigi and Wakiso and the least common was cream isolate obtained from Wakiso. Out of all the seven field isolates from six farms two isolates were confirmed to be pathogenic. Cream re-isolate originally sampled from plants exhibiting collar rot in Wakiso/Kawanda was positive for three tests i.e.; selective medium test on PCNB, *Fusarium* like macro and microconidia (Leslie, 2006) were visible under a microscope at and confirmed pathogenic because it was re-isolated from diseased P. edulis with an MDI of 3.3 an indication that the plant is susceptible to an infection from the above fungus and specificity because it was not pathogenic to *Solanum lycopersicum* (tomatoes) which had an MDI 1.2 and *Solanum anguivi*.Lam (African eggplant "katunkuma" Luganda) which had an MDI 1.3 (results not shown). The cream re-isolate from Wakiso was identified as *Fusarium* solani because it was amplified by TEF-Fs4 primer for TEF-1α gene specific to *F. solani* (Arif et al, 2012). Purple re-isolate originally sampled from plants exhibiting wilt symptoms in Mpigi was positive for three tests i.e. on the selective medium PCNB , *Fusarium* like macro and microconidia were visible under a microscope and confirmed pathogenic because it was re-isolated from diseased *P. edulis* (yet initially isolated from *Passiflora* maliformis) with an MDI

of 2.2 and specific pathogenicity to passion fruit as it was re-isolated from only them among all the test plants i.e. *Solanum lycopersicum* MDI 1.1 and *Solanum anguivi* Lam MDI 1.2 (results not shown). Results are further justified as significant by ANOVA at a mean of 2.274b at a $p<.001$. The above isolate was identified as a *Fusarium* because it was amplified by TEF-Fu3 primer which is specific for TEF-1α gene in *Fusarium* sp. (Arif *et al.*, 2012).

Pathogenicity of the cream re-isolate is in agreement to a quotation from Fischer (2008) "despite *F. solani* being a polyphagous agent affecting great variety of plants, studies in Taiwan show that *F. solani* in passion fruit plants is a specialized genus adapted to *Passiflora* sp. The results showed that it was not pathogenic to *Solanum lycopersicum* (tomatoes) which had an MDI 1.2 and *Solanum anguivi.* Lam (African eggplant "katunkuma" Luganda) which had an MDI 1.3. Overall, the findings indicate that there is a limited number of fungal species causing collar rot i.e. *Fusarium* solani and wilt i.e. *Fusarium oxysporum* f.sp *Passiflora*e in passion fruit. Because fungal pathogens tend to be highly variable, an investigation into within species variability in relation to their pathogenicity is necessary.

6. Conclusion

The only Fusarium species among the isolates from Uganda causing wilt was Fusarium oxysporum f.sp Passiflorae, and that for collar rot was Fusarium solani. Therefore, collar rot and Fusarium wilt in central Uganda are each caused by specific strains of Fusarium pathogens. The identification of pathogenic Fusarium in farmers' orchards is a starting point for designing effective disease management measures against the predominant fungal pathogenic variants in passion fruits. Follow-up wide-scale surveys in other regions are required for detailed analysis of fungal variates which devastate passion fruits if lasting strategies for effective control are to be realized.

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7.2. Conflict of interest

The authors declare that they have no conflict of interest. This research did not involve human and/or animal participants. The plant samples for disease diagnosis were collected from farmers' fields upon informed consent to the farmers and after approval by the College of Veterinary Medicine, animal resources, and bio-security (COVAB) of Makerere University.

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