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Characterization of *Fusarium* spp associated with mango malformation diseases in the South of Senegal

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Abstract

Mango is a fruit with huge economic importance in Senegal, as in many tropical and subtropical countries in the world. Its production and trade increased tremendously since exportation started in 1997. However, mango diseases like floral and vegetative malformation is one of the most important constraints causing serious economic losses. Several species of the *Fusarium* genus have been associated to this disease. In Senegal, only one study has been carried out on mango malformation diseases in the south and *Fusarium tupiense* was identified as the causal agent. The present study was undertaken to explore the diversity of strains/species of the causal agents of mango malformation diseases in Senegal. Strains of *Fusarium* spp isolated from malformed mango flowers of different trees, collected in different locations in southern Senegal were characterized on the basis morphological criteria and using molecular biology. The ITS region from the ribosomal DNA was targeted for the molecular biology analysis. The morphological characterization brought out a diversity of *Fusarium* structures associated with mango malformation suggesting diverse species. The molecular characterization allowed a better refinement, confirming the diversity of species without leading to a definitive specific identification. Therefore, a molecular characterization with specific primers should be considered.

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Introduction

Mango is one of the most important fruit crops grown in many tropical and subtropical regions around the world (Iqbal *et. al.,* 2005). Its yearly production is estimated to reach 33 million tons (Planetoscope, 2018). Almost 4% of this production comes from West Africa (Le citoyen, 2017) where Senegal is one of the main exporting countries. In this country, mango is the main fruit crops (Rey, 2011) with production feeding into food security but also holding a prominent role in exportation. In 2017, a total volume of 17,168 tons was exported, corresponding to a monetary value of 10 billion CFA francs (CIR, 2017). While In 2021, a volume of 24500 tons was exported of which 80% to Europe (Dieye *et. al.,* 2021). However, mango production is facing numerous phytosanitary constraints related to diseases and pests. Many losses are caused by fruit flies with damages reaching 74% in specific agro systems (Diédhiou *et. al.,* 2014) Mango anthracnose is the far most destructive mango pest in the rainy season in the south in particular where 90% to 100 % of the mango in orchards can be infested (Diédhiou *et. al.,* 2014). The mango malformation disease has added itself to these phytosanitary constraints more recently. This disease was detected in 2009, in southern Senegal for the first time (Sengor *et. al.,* 2012). It has been reported to have a potential destructive capacity, with losses in mango production of over 98%, making it a big concern beside anthracnose and the fruit fly (Sylla, 2010). Geographic variation in the incidence and severity of mango malformation was observed in different districts in southern Senegal (Dieye *et. al.,* 2021). Sedhiou was considered as the origin of the disease with an incidence of 91.94% and a severity of 55% (Dieye *et. al.,* 2021). The vegetative and floral malformation of the mango tree is found in several mango-producing countries where it was rated as one of the most important diseases attacking this speculation (Youssef *et. al.,* 2006).

Mango malformation disease was first reported to India in 1891 (Marasas *et. al.,* 2006). Since then, it was also reported in many countries in Asia, Africa and America (Freeman *et. al.,* 1999; Britz *et. al.,* 2002; Marasas *et. al.,* 2006). Its damages range from malformation of inflorescences to the lack of fruit setting (Freeman *et. al.,* 1999). Mango tree malformation disease manifests itself as a vegetative malformation, particularly in young plants in nurseries (Marasas *et. al.,* 2006) but also in adult plants (Otero-Colina *et. al.,* 2010). Infection of the floral tissues causes the inflorescences to be stunted, thick, bifurcated. It also increases the number of flowers and their size and induces sterility or abortion of the remaining hermaphrodite flowers. In addition, leaves can develop inside the inflorescences to become phyllodes (Marasas *et. al.,* 2006). Many biotic and abiotic factors were put in association with this disease (Ansari *et. al.,* 2015). Among these factors, fungi have been often reported to be the main cause (Kumar *et. al.,* 2016). A number of species of the genus *Fusarium* are associated with the malformation of the mango tree worldwide (Senghor *et. al.,* 2012; Dieye and 2021). *Fusarium tupiense* was reported as a pathogen responsible for the malformation of mango trees in southern Senegal (Senghor *et. al.,* 2012). However, the current level of spread of the disease in this part of the country in particular, and the numerous reports pointing at different associations of *Fusarium* species make it worth to scrutinize on the identity of the causal agent of the mango malformation in Senegal.

The objective of this work is therefore to gather more information on the identity of the fungal agent causing mango malformation in southern Senegal.

Materials and methods

Sampling

Samples of malformed mango inflorescences were taken from trees in villages located along the roads in different districts of the southern regions (Kolda, Sedhiou and Ziguinchor) of Senegal (Table 1). The major East to West road axis was, from Tambacounda to Kolda, then from Kolda to Sedhiou and further to Ziguinchor. On the North to South road axis, sampling took place between Ziguinchor and Bignona and further to Diouloulou next to the border to the Republic of Gambia. Also departing from Bignona, the South to North axis to Carrefour Diaroumbé and

Eastwards to Kolda was sampled (Fig. 1). Sample was collected in villages separated by at least 30 km.

Sample distribution was related to the incidence of the disease in the different districts as shown in Table 1.

Table 1. Origin of strains of *Fusarium* spp.

Strain code	Location	District
VDL2J (I)	Diouloulou	Bignona
Bignona S2	Bignona	Bignona
Bignona S2 (2)	Bignona	Bignona
Bignona S2 (3)	Bignona	Bignona
MCD S3	Diaroumé	Sedhiou
MCD S3 (1)	Diaroumé	Sedhiou
MD (I)	Dabo	Sedhiou
11 MNK (4)	Manguier Mankono Ba	Sedhiou
11 MNK (5)	Mankono Ba	Sedhiou
5 MNK (2)	Mankono Ba	Sedhiou
MCRZ S5	Kolda	Kolda
MCRZ S5 (5)	Kolda	Kolda
MCRZ S5 (3)	Kolda	Kolda
MCRZ S2	Kolda	Kolda
MSM S2	Saré Mady	Kolda
9 MCRZ (I)	Kolda	Kolda
9 MCRZ	Kolda	Kolda
MSM S2 (1')	Saré Mady	Kolda
MSM S2 (2')	Saré Mady	Kolda

Fig. 1. Administrative map of the southern region of Senegal with the sampled districts for mango malformation disease.

Isolation and purification

The samples (Floral and vegetative malformations) were first disinfected with 1%. Then, using a sterile scalpel blade, explants were taken. Under the fume hood, these explants were successively immersed in 70% alcohol, rinsed with sterile distilled water, wiped with sterile absorbent paper and finally inoculated with 3 explants per Petri dish containing the PDA medium. The plates are incubated in the oven at 25°C. Few days after, some of the mycelium is transferred to other plates to obtain pure strains.

Single-spore culture protocol

Single spore isolate was carried out using a protocol to obtain genetically homogeneous fungal material as described by Booth (Booth, 1971). For this purpose, the isolates were first transplanted to Petri dishes containing potato dextrose agar medium and allowed to grow over the entire surface of the dish for 7-10 days. A 5mm diameter mycelial disc was taken from the periphery and introduced into a tube containing 9ml sterile distilled water. After shaking, dilutions to one hundredths are made with a sterile pipette. This operation is repeated as many times as necessary until the desired dilution is reached. Then a volume of 0.2ml generally containing 2 to 10 microconidia adjusted with the Malassez cell was taken from the last 2 dilutions and spread with sterile beads on a 2% agar medium. Identification and delimitation of germinating spores was performed using a binocular magnifying microscope after 24-48h incubation at 25°C. Fragments of 3 to 4 of these conidia are collected with their agar using a sterile tooth pick and placed in Petri dishes containing BLA for incubation at 25°C for one week. This operation was repeated for all the isolates.

Morphological features

To study the morphological variation between different *Fusarium* spp. isolates, each isolate is transferred to a petri dish containing a PDA medium combined with chloramphenicol to avoid bacterial contamination. The inoculation was done using a 5mm diameter mycelial disc taken aseptically with a fume hood punch from a 10-day old *Fusarium* sp. culture before incubation at 25°C. The daily evolution of the mycelial diameter of the different isolates was regularly done for each replicate. The mycelial diameter was measured on the horizontal and vertical planes of the box and the average of the two measurements was calculated. This procedure was carried out until the mycelium had grown over the entire surface of the box.

The macroscopic study was done based on the color on the surface and reverse side of the boxes, the growth rate of the mycelium and the pigmentation. While the microscopic description was done by

observing the shape of the microconidia, macroconidia, phialides and chlamydospores. In addition, a biometric study was carried out on 50 conidia per isolate.

Molecular characterization

Molecular identification was done using the regions of the internal transcribed spacer. DNA was extracted from mycelia grown on Petri dishes containing potato dextrose agar (PDA) medium.

DNA extraction was performed according to the method described by Aamir *et al.* in 2015 modified (Aamir *et. al.,* 2015). The *Fusarium* spp strains were first refreshed in Petri dishes containing P.D.A. medium and amoxicillin. About ten days later, for each strain, a portion of the mycelium was excised with a scalpel blade and then cut into small fragments and introduced into a sterile Eppendorf tube containing sterile glass beads. In each tube, 800 µl of lysis buffer (Tris HCl, EDTA, SDS) was added and the fragments were then crushed using a plastic pestle. All the tubes were then vortexed for 5 min and placed in a water bath at 65°C for 30 min. This was followed by centrifugation at 6000 rpm for 15 min. The supernatant was introduced into a new sterile Eppendorf tube. A volume of 2µl of RNase A was then added to each tube and were incubated in a water bath at 37°C for 15 min. An equal volume of phenol chloroform isoamyl alcohol (25: 24: 1) was added to the contents of each tube before vortexing and centrifugation at 6000 rpm for 15 min. The supernatant was collected and introduced into a new, fresh sterile Eppendorf tube. An equal volume of pure ethanol was then added to the contents of each tube and all tubes were vortexed and then incubated for 1h at -20°C. After incubation, the tubes were centrifuged at 6000 revolutions/min for 15 min. The supernatant from each tube was discarded and 800 µl of 70% ethanol was added to wash the pellet. This was followed by a new centrifugation at 6000 rpm for 10 min. The ethanol was then poured in and the resulting pellet corresponding to the DNA was then dried using a "Speed Vac". After drying, 50µl of 1X TE buffer was introduced into each tube and then all the tubes were sealed and then stored at -20°C.

A PCR reaction was prepared for all 19 DNA samples according to the following protocol using ITS1 / ITS4 primers and reagent mixture shown in Table 2. The PCR was performed with a ABI "Verity" thermocycler using to the following program: 95°C, 3min; (95°C, 1 min; 58°C, 1min; 72°C, 10 min) 35x; 72°C, 10 min.

Table 2. Reagents for the PCR reaction for a total volume of 25 µl par sample.

Reagents	Quantity for 1 tube (µl)
H_2O	11,45
Buffer 5X	5
dNTPs (2,5 mM)	1
$MgCl_2$ (25 mM)	2,5
ITS 1 (20 µM)	0,625
ITS 4 (20 µM)	0,625
BSA (10 mg/ml)	0,5
Go Taq	0,3
ADN (1/20)	3

PCR products were detected by migration electrophoresis on 1% agarose gel in the presence of a 1Kb molecular weight marker. The photos were visualized by the photo documentation system Gel-Doc under 360 nm UV light. The PCR products from the amplified DNA samples were sent to the sequencing laboratory for further analysis.

After DNA sequencing, quality was checked using the BioEdit software and sequences were compared by Standard Nucleotide Basic Local Alignment Search Tool (BLASTn) with those of the database of the National Center for Biotechnology Information (NCBI). The sequences were then aligned with sequences from the NCBI database with ClustalW using the BioEdit software. A phylogenetic tree was then constructed using MEGA 5.05 software (Tamura *et. al.,* 2011) according to the Maximum Likelihood algorithm algorithm with 1000 bootstrap replications (Felsenstein, 1985). The identification of each isolate was defined based on the closest percentage resemblance to the Blast results. The resulting sequences were subsequently submitted to the NCBI for accession number assignment.

Results

Morphologic features

The different strains of *Fusarium* spp showed a variety of structures. Macroconidia (Fig. 2a and 2b),

microconidia (fig. 2c and 2d) and monophialides (fig. 3a) were present in all strains. Polyphialides were only visible in some strains (MCRZ S2, MSM S2 (1 '), MSM S2 (2'), 9 MCRZ, 9 MCRZ (I), MCRZ S5 (3), MD (I), MCD S3, 11 MNK (5)) (fig. 2b) as well as chlamydospores (MCRZ S2, Bignona S2 (3), MSM S2 (2 '), MCD S3 (1), 5 MNK (2), 9 MCRZ (I), MCRZ S5 (3), 11 MNK (4), MD (I), MCD S3, 11 MNK (5)) (fig. 3: c, d, e). In addition to these structures, chains of microconidia (5 MNK 2, 9 MCRZ, Bignona S2 (2), Bignona S2 (3), MCD S3 (1), MCRZ S5, MSM S2, MSM S2 (2 ')) (fig. 4a) as well as spiral hyphae (VDL2J (I), MSM S2, MSM S2 (2 '), MSM S2 (1'), MSM S2 (1), MD (I), MCRZ S5, MCRZ S5 (3), MCRZ S2, MCD S3 (1), Bignona S2 (2), 11 MNK (5), 11 MNK (4), MSM S2 (2 ')) (fig. 4b) were observed for certain strains.

The characterization of the *Fusarium* spp strains showed that some of them had similarities with species associated with the mango malformation. Strains VDL2J (I), MSM S2 (1), MCRZ S5, MSM S2, showed more resemblances to *F. sterilihyphosum*; Bignona S2, Bignona S2 (2) and 9 MCRZ (I) with *F. proliferatum*; MCRZ S5 (5) with *F. mangiferae*. The rest of the strains, which produced chlamydospores unlike the previous ones and *Fusarium* spp species associated with the mango malformation, were not identified with any particular species (Table 3).

Table 3. Morphologically associated species for the strains of *Fusarium* spp studied and degree of similarity with the reference species from the NCBI database.

Strain	District of origin	Associated species according to morphological criteria	Similarity after sequence analysis (%)	Associated reference species from NCBI database
VDL2J (I)	Bignona	*F. sterilihyphosum*	99%	
MCD S3 (1)	Sedhiou	None	99%	*Fusarium fujikuroi* strain
Bignona S2	Bignona	*F. proliferatum*	99%	O6-2
9 MCRZ (I)	Kolda	None	99%	
MSM S2 (2')	Kolda	None	99%	*Gibberella moniliformis*
MCRZ S5 (5)	Kolda	*F. mangiferae*	99%	clone 11
5 MNK (2)	Sedhiou	None	99%	
MCD S3	Sedhiou	None	99%	*Gibberella moniliformis*
11 MNK (5)	Sedhiou	None	99%	strain XM060107
11 MNK (4)	Sedhiou	None	99%	
MCRZ S2	Kolda	None	99%	
MCRZ S5 (3)	Kolda	None	99%	*Fusarium pseudonygamai* isolate wxm62
9 MCRZ	Kolda	*F. proliferatum*	99%	*Fusarium proliferatum* strain NBt7C1
MCRZ S5	Kolda	*F. sterilihyphosum*	99%	*Fusarium oxysporum*
MSM S2 (2')	Kolda	None	99%	isolate MM2-EGY
Bignona S2 (2)	Bignona	*F. sterilihyphosum*	100%	*Gibberella intermedia* strain LVPEI.H860_10
MCD S3 (1)	Sedhiou	None	99%	
Bignona S2 (3)	Bignona	None	99%	*Fusarium sp*. AS 407
MD (I)	Sedhiou	None	99%	
MSM S2 (1')	Kolda	*F. sterilihyphosum*	99%	*Fusarium sp*. MT2S520
Bignona S2 (2)	Bignona	*F. sterilihyphosum*	100%	
MSM S2	Kolda	*F. sterilihyphosum*	99%	*Fusarium sp*. E109
Bignona S2	Bignona	*F. proliferatum*	99 %	
11 MNK (4)	Sedhiou	None	99 %	
9 MCRZ (I)	Kolda	None	99 %	

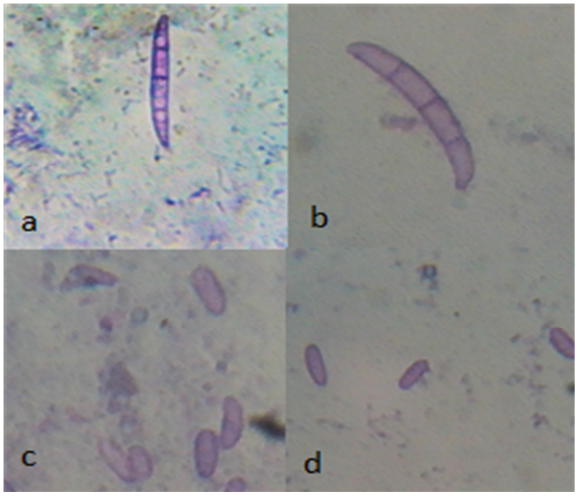

Fig. 2. a and b) Macroconidia; c and d) Microconidia (1000 X).

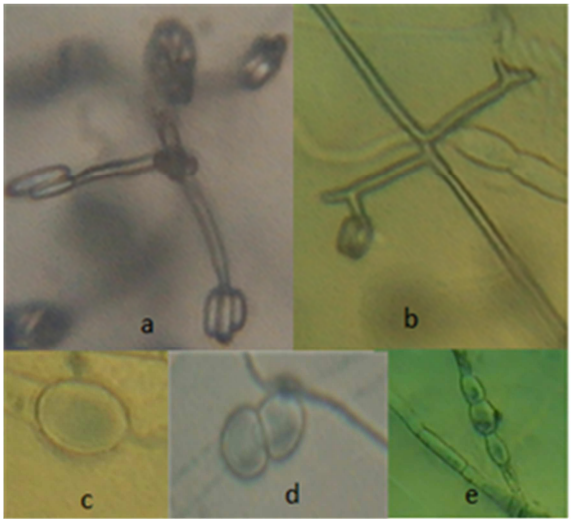

Fig. 3. a) Monophialides; b) Polyphialides; c) Single chlamydospore; d) Paired chlamydospores; e) Chlamydospores in chain (1000 X).

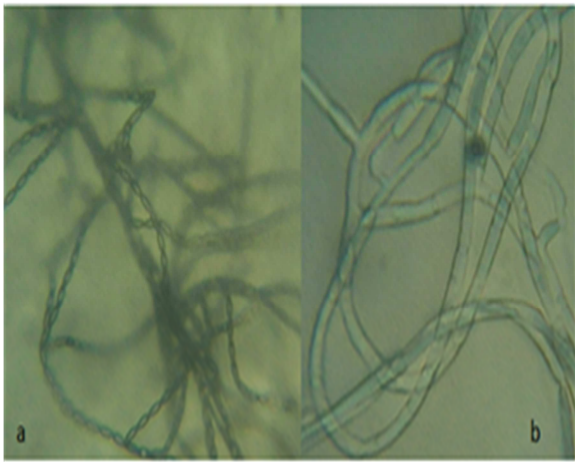

Fig. 4. a) Chains of microconidia; b) Spiral hyphae (1000 X).

Molecular characterization

Amplification of the ITS region of ribosomal DNA from *Fusarium* spp. strains with the ITS 1 and ITS 4 primers resulted in bands of sizes between 500 and 600 base pairs. The results of sequences BLASTn alignment for species identification gave the results in Table 3. The strains VDL2J (I), MCD S3 (1), Bignona S2 and 9 MCRZ (I) exhibited 99% similarity with the O6-2 strain of *F. fujikuroi* of NCBI database. The MCD S3 strain (1) also showed 99% similarity to *Fusarium* sp. AS407, as well as *Bignona* S2 (3) and MD (I). The strains MSM S2 (1 ') and *Bignona* S2 (2), showed, respectively, 99% and 100% similarity with *Fusarium* sp. MT2S520. MSM S2 (2 ') and MCRZ S5 (5), showed 99% similarity to *G. moniliformis* clone 11. The MSM S2 (2 ') strain showed 99% similarity with the MM2-EGY strain of *F. oxysporum*, as did MCRZ S5. The strains 5 MNK (2), MCD S3, 11 MNK (5), 11 MNK (4) and MCRZ S2, showed 99% similarity with the XM060107 strain of *G. moniliformis*.

MCRZ S5 (3) strain showed 99% similarity with *F. pseudonygamai* isolate WXM62 while 9 MCRZ showed 99% similarity with *F. proliferatum* strain NBt7C1. MSM S2 exhibited 99% similarity with *Fusarium* sp. E109. The *Bignona* S2 (2) strain also exhibited 100% similarity with the LVPEI.H860_10 strain of *G. intermedia*. In addition, the strains VDL2J (I), 9 MCRZ (I), Bignona S2 and 11 MNK (4), also showed 99% similarity with the strain FK070205 of *G. fujikuroi*.

The phylogenetic tree (Fig 3) shows two large groups

The first group is made up with two subgroups. The first subgroup with a bootstrap value of 65%, is formed by all the strains of *Fusarium* spp studied, with the exception of MSM S2 (1'), MSM S2 and Bignona S2 (2), as well as the reference species *F. fujikuroi* strain O6-2 from the NCBI database. The second subgroup consists of *G. moniliformis* strain XM060107, *Fusarium* sp. AS407, *F. proliferatum* strain NBt7C1, *F. oxysporum* isolate MM2-EGY, *G. moniliformis* clone 11, *F. pseudonygamai* isolate wxm62, from the NCBI database.

The second group, with a bootstrap value of 97%, consists of two subgroups; one of which is formed by

Fusarium sp. E109 from the NCBI database, while the second subgroup, with a bootstrap value of 62%, is composed of three strains MSM S2 (1 '), MSM S2 and *Bignona* S2 (2) and two reference species from the database of NCBI (*Fusarium* sp. MT2S520, *G. intermedia* strain LVPEI.H860_10).

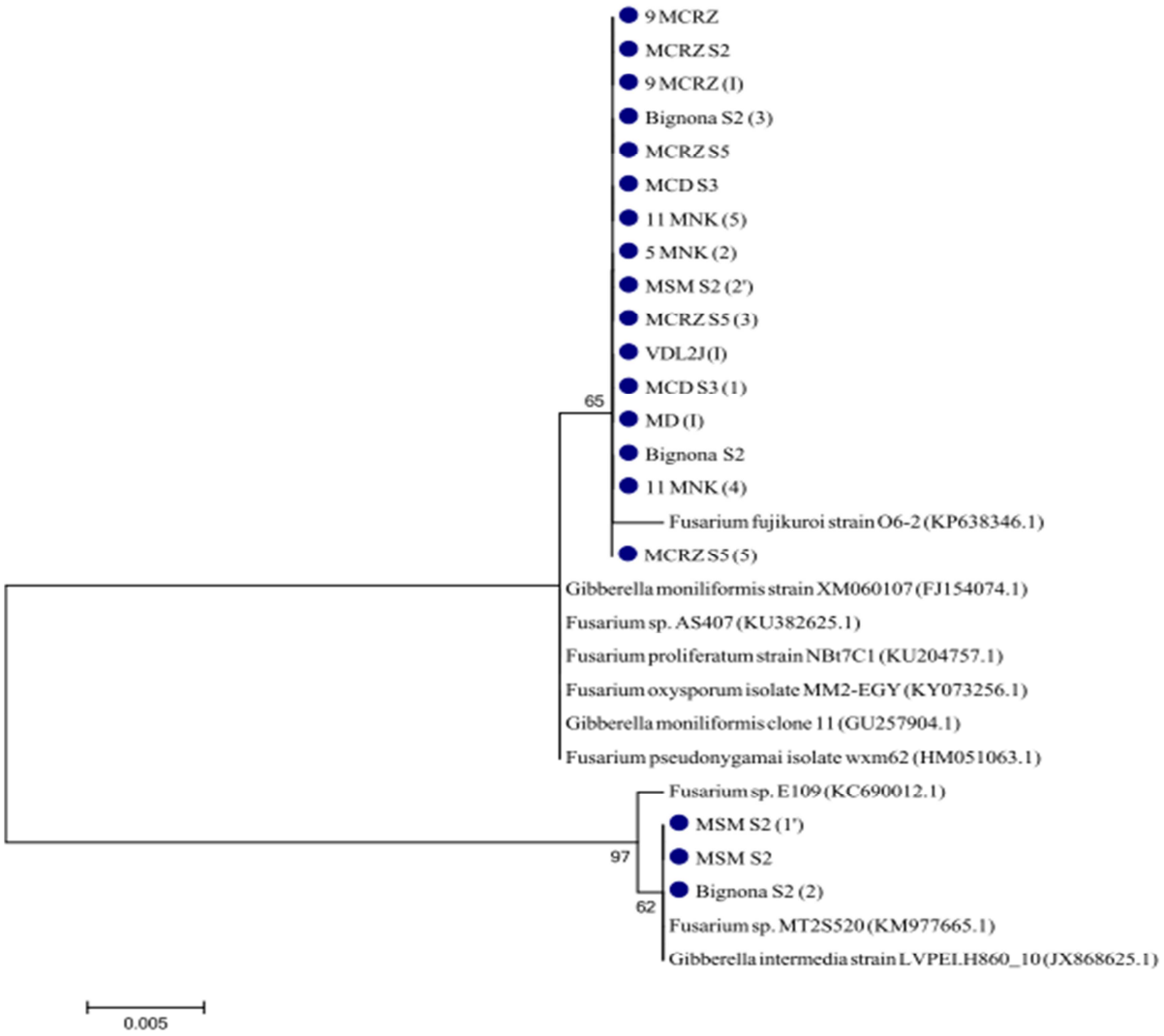

Fig. 3. Phylogenetic tree showing the relationships between the *Fusarium* spp. isolates studied based on ITS gene sequence using the Maximum Likelihood algorithm with 1000 bootstrap replications.

Discussion

A variety of fungal structures have been observed during the microbiological study of the strains isolated from malformed mango flowers. The structures encountered were macroconidia, microconidia, monophialides and polyphialides, that are typical of the genus Fusarium, in particular species associated with the malformation of the mango tree (Britz *et. al.,* 2002; Leslie and Summerell, 2006; Otero-Colina *et. al.,* 2010; Senghor *et. al.,* 2012). These results are in line with those of Dieye *et al.,* 2021 who worked on the characterization of *Fusarium* associated with mango malformation in Senegal (Dieye *et. al.,* 2021). These results confirm that the strains studied belong to one or more species associated with the malformation of the mango tree in the southern zone of Senegal. Other structures more specific to certain species associated with this disease have also been observed. Those concerned chains of microconidia associated with *F. proliferatum*, *F. pseudocircinatum* and spiral hyphae that are related to *F. pseudocircinatum* (Leslie and Summerell, 2006), *F. mexicanum* (Otero-Colina *et. al.,* 2010), *F. sterilihyphosum* (Britz *et. al.,* 2002).

The use of morphological criteria has made it possible to make a provisional identification that reveals strains showing similarities with the species *F. sterilihyphosum*, *F. proliferatum*, *F. mangiferae* and other strains not associated with a particular species. Dieye *et al.* associated morphologically the strains from that area to 5 species namely *F. subglutinans*, *F. oxysporum*, *F. sterilihyphosum*, *F. equiseti* and *F. mangifera*e (Dieye *et. al.,* 2021). The use of sequence alignment showed that the strains VDL2J (I), Bignona S2, 11 MNK (4) and 9 MCRZ (I) displayed 99% similarity with the FK070205 strain of *G. fujikuroi*. However, *G. fujikuroi* is the teleomorph of a number of species, including *F. fujikuroi* (Leslie and Summerell, 2006). In addition, the 9 MCRZ and Bignona S2 strains (2) showed high level of similarity with the reference species from NCBI, respectively *F. proliferatum* strain NBt7C1 (99%) and the LVPEI.H860_10 strain of *G. intermedia*, which is the teleomorphic stage of *F. proliferatum* (Leslie and Summerell, 2006). In most cases, the corresponding reference species do not coincide with those retained as similar during the morphological characterization, which shows the limits of the latter. Among the identified reference species, *F. proliferatum* is known as a pathogen responsible for mango malformation disease (Freeman *et. al.,* 2014).

These results confirm that mango malformation disease in southern Senegal is associated with a species complex of the genus *Fusarium* of the Gibberella section as already reported by Dieye *et. al.,* 2021 (Dieye *et. al.,* 2021).

Among the strains studied, four showed 99% similarity with the strain FK070205 of the teleomorph *G. fujikuroi*. It seems therefore interesting to see more closely the relationships between the strains of *Fusarium* spp isolated from southern Senegal and this teleomorph, since a good number of *Fusarium* species identified as responsible for the malformation of the mango tree belong to the species complex *G. fujikuroi*. Among these species *F. mangiferae*, (Britz *et. al.,* 2002), *F. sterilihyphosum* (Britz *et. al.,* 2002), *F. tupiense* (Lima *et. al.,* 2012; Freeman *et. al.,* 2014), *F. mexicanum* (Otero-Colina *et. al.,* 2010) can be mentioned. Furthermore, the strains of our study did not appear to be intimately related to the NCBI reference species on the phylogenetic tree.

Ultimately, in view of the results obtained with the use of molecular biology tools, it does not seem possible to identify exactly all the *Fusarium* spp species corresponding to the strains studied at this level, although this made it possible to gain in precision. This could be linked to the fact that the primers used are not specific and therefore do not always make it possible to distinguish very close species due to the variability of the ITS zones (Moine, 2013). Therefore, it would be interesting to proceed to a more acute refinement of the approach by testing specific primers of the closest species to the strains, on the one hand. On the other hand, molecular identification could be based on genes well represented in databases such as those for β-tubulin and elongation factor -1α (tef), because these are well conserved (Moine, 2013). It seems however clear that the mango malformation in southern Senegal is most likely to be caused by a combination of *Fusarium* species rather than a single one. This finding confirms the results of Dieye *et al.,* who associated mango malformation disease with 08 *Fusarium* species namely *F. subglutinans*, *Gibberella moniliformis* strain EXGF-2, *F. sterilihyphosum* isolate EFA 6FSRG, *F. verticillioides* strain AE-FPO8, *F. circinatum*, *F. sudanense*, *F. proliferatum* TF1 and *F. equiseti* (Dieye *et. al.,* 2021).

Conclusion

In the present study, the results obtained indicate the probable involvement of several *Fusarium* species in mango malformation. However, they did not allow identification to the species level of the *Fusarium* spp. strains studied. Morphological identification could be focused on more stable and obvious criteria. Molecular characterization showed that despite the high degree of similarity noted between the strains studied and the NCBI reference strains, identification to the specific level requires additional tests. It would therefore be important to continue with specific primers in order to be definitive about the species involved.

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