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Molecular Identification of Fungal Pathogens Associated with Post-harvest Yam Tubers Rot in Mbam et Kim Division (Cameroon) with Emphasis on *Penicillium monomenatosum* (Frisvad, Filt. & Wicklow) as a First Report

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Abstract Nowadays, increasingly complains mention tremendous decay of stored yam tubers in the Mbam et Kim Division, the main producing zone of Cameroon. Knowledge of the specific fungi responsible for yam spoilage is paramount for proper preservation by implementing plausible control measures. This study was therefore undertaken to identify the causative fungal agents associated with yam tubers rot in the survey zone. The isolation of suspicious pathogens was performed as per the Koch's postulates encompassing artificial inoculation, symptoms recording, re-isolation and comparisons-based re-identification. The identity of isolates was later on ascertained by the sequencing of the 5.8S ribosomal DNA gene (ITS1 and ITS2). Overall, seven fungal species: *Fusarium solani*, *Fusarium oxysporum*, *Aspergillus niger*, *Penicillium monomenatosum*, *Rhizopus oryzea*, *Talaromyces flavovirens* and *Bionectria cf. ochroleuca* were recovered. *P. monomenatosum* is reported here for the first time as causative agent of yam rot. The obtained results unveil the identity of the devastating fungus threatening yam availability to consumers throughout the year in Cameroon and represent an initial and crucial step toward planning efficient and reliable control measure to counter the yam rot epidemic.

Keywords: *Mbam et Kim division, fungi, yam tubers rot, Penicillium monomenatosum*

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1. Introduction

Yams (*Dioscorea* spp) are very important staple foods in tropical and subtropical regions, contributing to sustainable livelihood of millions farmers [1]. In 2012, the Food and Agriculture Organization of the United Nations (FAO) estimated to about 56,000 million tonnes of yam tubers produced worldwide, of which about 95% is from Africa. Cameroon ranks seven among yams producing countries with *D. rotundata* and *D. alata* the most cultivated and consumed species [2].

Yams-based foods are endowed with high nutritional values because of their richness in carbohydrates, proteins and dietaryfibers [3,4]. *Dioscorea* tubers are good diet enrichments and also ethno-medicinally important, owing to their outstanding antioxidant properties [5,6,7]. In most African countries including Cameroon, yams bear beyond their market values, cultural, religious, and social meanings, which vary between specific ethnic groups and regions [8].

It is well-recognized that the actual national yams production is unable to satisfy the increasing local populations. Limitations like wrong cultural practices, coupled to pests and pathogens attack are key factors

hindering the productivity of this staple both in farms and storages units [9]. In fact, for permanent availability of yam tubers to consumers throughout the year, many farmers rely on traditional methods for post-harvest preservation [10], which unfortunately, are not suitable for long lasting storage because they often fail to protect a great proportion of tubers as pathogen's ingresses oftentimes result in huge losses [11]. Depending on the pathogen, infected yams tubers may display dry rot, soft rot and wet or watery rot, with straight link with their putative physio-pathology [12]. Therefore, the diagnosis of the disease along with the identification of the proper causing agent is a critical step prior to designing appropriate mitigation measures or to completely eradicate the threats so as to insure the availability of tubers to consumers throughout the year.

Fungi species belonging to diverse genera have been ascribed to yam tubers rot .Regardless of the geographic locations where yam decay has been reported, species belonging to Aspergillus, Botryodiplodia, Fusarium, Penicillium, Trichoderma, Rhizopus, Colletotrichum, Cladosporium, Cylindrocapus, Gliocladium, Geotrichum, Gliomatrixcon, Macrophomina, Mucor, and Rhizoctonia genera have been incriminated, causing as high as 80% annual yields decline in storage barns [13-18]. For instance, in our preliminary investigation undertaken in storage units in Yaoundé, we identified two pathogens namely *Fusarium solani* and *Rhizopus stolonifer [18]*. Although very preliminary, this finding provided an overview of potential yam rot causing agents in few localities of Cameroon. However, nothing is known about the extent of the disease and causing agents in the main yam producing basin of the Mbam et Kim Division. To answer above questions in response to numerous farmers' complains for tremendous yam tubers decay in storage, a survey was undertaken and a preliminary investigation led to the presumption of invasive and highly virulent fungal agents. The present paper describes among other fungi, the first report of *Penicillium monomenatosum* as causative agent of postharvest rot of yam tubers.

2. Materials and methods

2.1. Collection of Infected Yam Tubers

Both visibly rotted and healthy yam tubers were collected from different sales points in three villages market in yam-growing districts of the Mbam et Kim Division during June 2016. The samples were transferred into separate labeled sterile plastic bags to the laboratory for fungal isolation.

2.2. Fungal Pathogens Isolation and Taxonomic Characterization

Yam tubers showing rotting symptoms were washed with running tap water to remove soil particles. Each tuber was surface sterilized in10% sodium hypochlorite for 5 minutes and rinsed thrice with sterile distilled water. Thereafter, 3 to 4 mm yam pieces picked from rotting edge with a sterilized forceps were inoculated onto potato dextrose agar medium supplemented with chloramphenicol. Plates were incubated at 28 ± 2°C and checked daily for seven days. Any emerging microorganism was sub-cultured on fresh PDA plate to obtain pure cultures. Thereafter, seven days old isolates representing all the recovered morphotypes were prepared in lactophenol cotton blue and mounted on light microscope for preliminary identification. Characteristic like hyphae type (septated or not), conidiophores or phialides structures, conidiogenous cells, and specific reproductive propagules were used to identify the isolates following specific taxonomic identification keys [32] Isolates were later one .subjected to the pathogenicity test.

2.3. Pathogenicity Testing

To ascertain the virulence of the isolated fungi, a pathogenicity test was performed by axenic inoculation of fresh and healthy yam tubers. Prior to the assay, healthy yam tubers were washed, surface sterilized, and blotted-dried [19]. Bore holes were then made into the tubers with a sterile cork borer [18]. Then, mycelia plugs (5 mm) taken from the edge of 5 days old cultures of each isolate were placed in the holes. Yam disk were thereafter used to cover the surface of the inoculation points alongside with wet cotton [19]. The inoculated tubers were incubated for fourteen days at 25±2°C. Regular observations were made during the incubation time to check for any sign of infection. The artificially induced symptoms were noted and compared to those recorded in farmer's yam barns. Similarly, the cultural and microscopic features of the original and the re-isolated fungi isolates from artificially infected tubers were compared in order to establish the patho-system and fulfill the Koch's postulates [32].

2.4. Molecular Identification of Fungi

After cultural and microscopic characterization of fungal pathogens, the sequencing of the ITS1-5.8S rRNA-ITS2 nucleotide sequence of ITS gene was used to confirm their respective identities [33]. Briefly, fungal DNA was extracted from 200 mg of mycelial mat scraped from a 3-day-old culture using a commercial isolation kit (PowerLyser^{R} PowerSoil^{R}DNA). The ITS1-5.8S rDNA-ITS2 region was amplified in a PCR mix made up of PCR buffer, deionized water, $MgCl_2$, dNTP, Taq polymerase, genomic DNA extract, and primers ITS3 (Forward primer) and ITS4 (Reverse primer). The thermal cycler (T3000, Biometra^{R}) was set at 95°C for 5 min (Initial denaturation), followed by a 35 cycles of 95°C for 5 min, 35 cycles of 95°C for 30 seconds min, 49°C for 30 seconds, and 72°C for 2 min after which the reaction was kept at 72°C for 5 min. PCR amplicons were purified using purification protocol with Nucleo Spin^{R} Gel and PCR Clean-Up kit and sequenced. The amplified ITS-rDNA gene were sequenced by BMC (Boulder Medical Center) Laboratory using the ITS3 primer.

The BLAST algorithm was used to find again similar sequences of those obtained from fungal isolates. The identification of fungal isolates was based on the similarity between fungal isolates sequences found and to those of reliable reference included in the NCBI Genbank public nucleotide databases. A dendrogram was drawn

with the nucleotide sequences of the isolates and those of reference strains deposited in Central Bureau voor Schimmelcultures (CBS), and American Type Culture Collection (ATCC) collections. Sequences alignment was performed using Clustal X 2.1, and the dendrogram was made using the neighbour joining method with Kimura 2-parameter distances by the MEGA 6.06 software. Alignment gaps were treated and considered as partial missing information. The robustness of the classifications was estimated by five hundred bootstrap replications. Groups of sequences gathered at close proximity within the same branch of the dendrogram were individually aligned. Sequences with a similarity greater than 98 % with reference sequence used for phylogenetic analysis were considered to belong to the same species as the reference sequence.

3. Results and Discussion

Yam is a very important staple food in Cameroon. Its availability to consumers is being seriously compromised because the storage in the main producing basins is suffering from the attack of several unidentified pathogens. In order to afford insight into etiology, as preliminary step towards mitigating measures, this study was undertaken to shed light on the yield-limiting pathogenic organisms prevailing in yams barns in the Mbam-et-Kim division, Cameroon.

A total of 87 fungal isolates were obtained from infected yams tissues with 54 (62.06%) and 33 (37.93%) respectively, from *D.rotundata* and *D. alata.* The high colonization rate was expected because yam's tissues are good growth medium to various microorganisms owing to their high nutrient and relative water contents [20,21]. Only 10 (11.50¨%) out of the 87 isolates could show clear physical evidence of spoilage upon pathogenicity assay as illustrated in Figure 1. The proportion of non-pathogenic agents obtained in this study is not surprising as reported earlier by Riley et al. [22]. In fact, the nutrient availability of decaying plants makes them favorable for the development of saprophytic organisms which are often confused with pathogens [22].

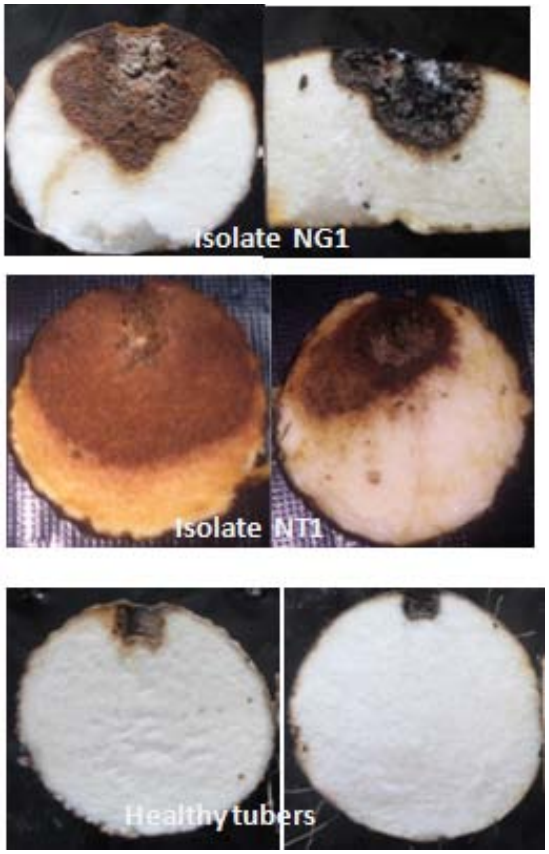

Figure 1. Yam tubers showing typical induced rotting symptoms by two fungi isolates

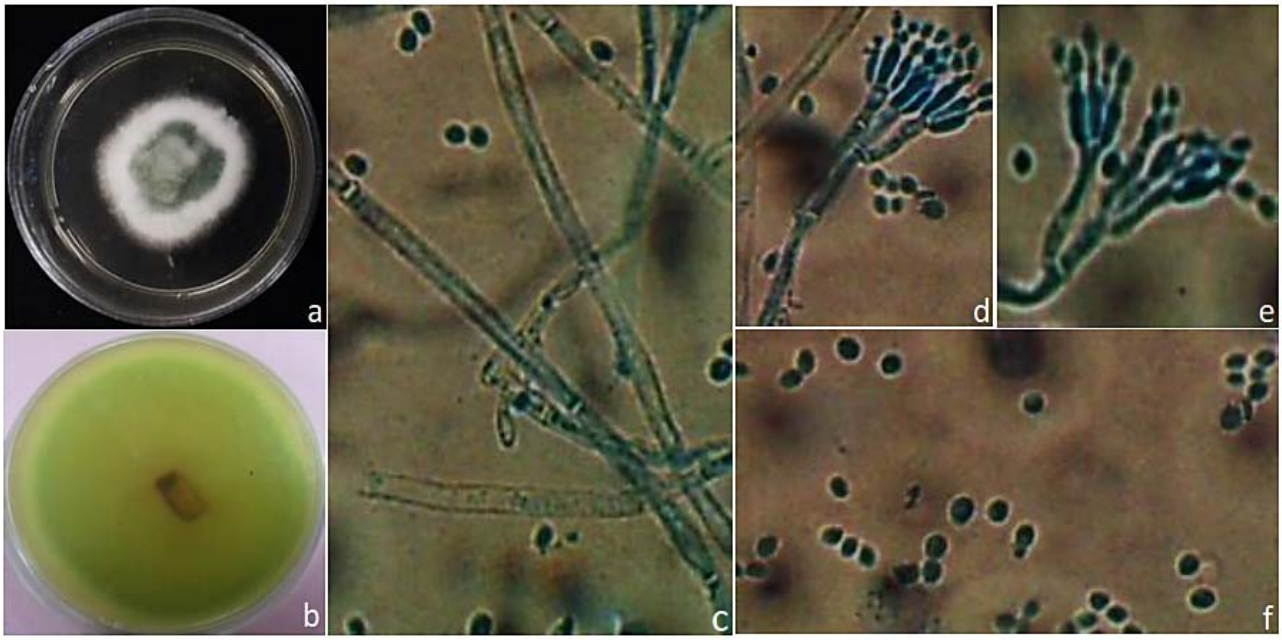

Figure 2. Cultural and microscopic characteristics of *P. mononematosum.* (a): general aspect of the colony on MEA, upon 3 days incubations; (b): yellow to green-yellow pigment excreted in the medium upon 7 days incubation at 28°C; (c): septated hyphae; (d) and (e): typical asymmetric terverticillate conidiphore with verticile phialides; (f): conidia

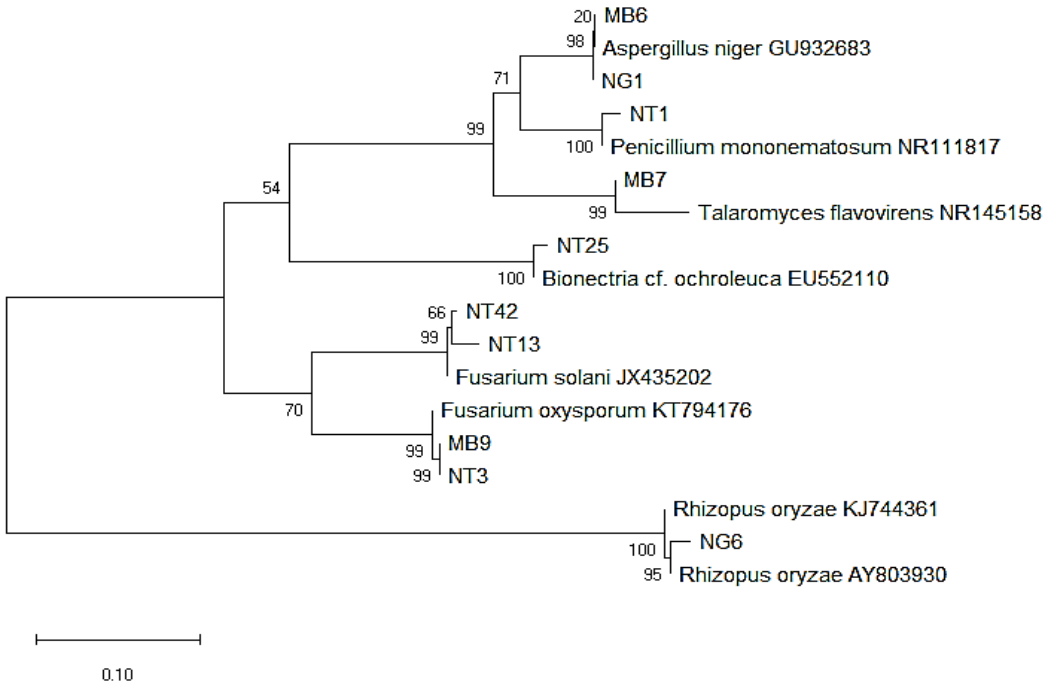

Figure 3. Phylogenetic tree showing relationship of Pathogenic fungi from Yam with other related fungal species. Sequence analyses were performed based on the rDNA gene sequence (ITS1-5.8S-ITS2) by Neighbor-joining tree model using "p-distance" for nucleotides with "the pairwise gap deletion" option. Numbers at branch nodes are bootstrap values, indicating support based on 500 replications

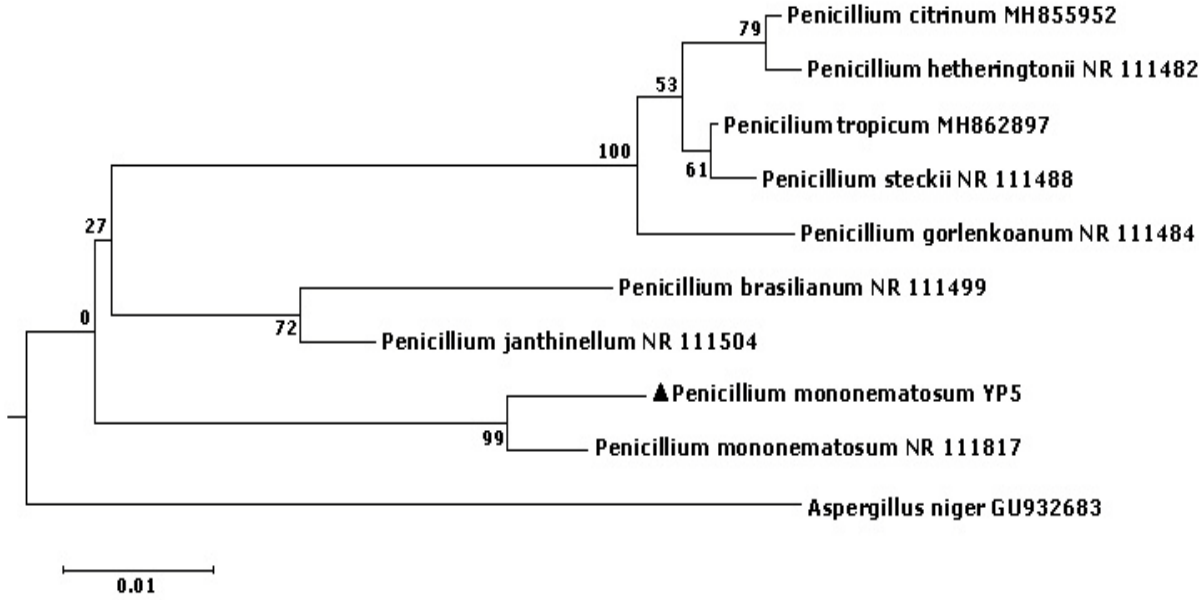

Figure 4. Phylogenetic tree showing relationship of *P. mononematosum.* with other related fungal species. Sequence analyses were performed based on the rRNA gene sequence (ITS1-5.8S-ITS2) by Neighbor-joining tree model using "p-distance" for nucleotides with "the pairwise gap deletion" option. Numbers at branch nodes are bootstrap values, indicating support based on 500 replications

Table 1. Iidentified fungal strains in yam tubers with NCBI gene bank

N°	Fungi code	Similarity percentage (%)	Organism with the highest sequence identity with GenBank Acc. no.
1	MB9	99	*Fusarium oxysporum* KT794176
2	NT13	99	*Fusarium cf . solani* JX435205
3	NT42	100	*Fusarium cf . solani* JX435202
4	NT3	99	*Fusarium oxysporum* KT794176
5	NG6	99	*Rhizopus oryzea* AY803930
6	NT25	99	*Bionectria cf. ochroleuca* EU552110
7	MB6	99	*Aspergillus niger* GU932683
8	NG1	99	*Aspergillus niger* AY939787
9	NT1	99	*Penicillium monomenatosum* NR111817
10	MB7	99	*Talaromyces flavovirens* NR 145158

The macroscopic and microscopic characterization of the ten pathogenic fungal candidates revealed that all belonged to Aspergillus, Bionectria, Fusarium, Rhizopus, Talaromyces (data not shown) and Penicillum (Figure 2) genera. Of note, the isolate NT1 suspected to be *Penicillum monomenatosum, was* never identified before as yam's pathogen. In fact, when grown on MEA, a standard medium recommended for the macro-morphological characterization of *Penicillium* spp., the colony of this isolate NT1 displayed white mycelium at the margins with glaucous grey to greenish grey colour at the middle marking with sporulation upon four days of incubation at 28°C. The colonies exhibited irregular-shaped concentric rings pattern with no radial sulcation (Figure 2a). More, NT1 produced yellowish to green-yellow strong soluble pigment in the medium seven days post incubation (Figure 2b). Microscopically, the mycelia were septate (Figure 2c) with terverticillate conidiophores branching patterns (Figure 2d-e), indicating two levels of branching between the stipe and the metulae, resulting in general asymmetrical layout. The phialides (42-13 µm) were vesiculate with swollen terminal cells. The conidia (Figure 2f) sizing 14 to 16 µm were transparent and abundant. Ultimately, the aforementioned characteristics suited perfectly with the previous description of *P. mononematosum* by Frisvad and Samson [26]. The sequencing of the ITS1-5.8S rDNA-ITS2 region of this isolate NT1 alongside with the nine other pathogens followed by phylogenetic analysis led to the confirmation of their identity as being *Fusarium oxysporum* (MB9, NT3), *Fusarium solani* (NT13, NT42), *Aspergillus niger* (MB6, NG1), *Rhizopus oryzea*, (NG6), *Talaromyces flavovirens* (MB7), *Bionectria cf ochroleuca* (NT25) (Figure 3 and Table 1), and *Penicillum monomenatosum* (NT1) (Figure 4 and Table 1).

Fungi belonging to the genera identified in the present study have frequently been reported as important rot pathogens associated with losses of yam in storage [18,23,24,25]. By providing evidences of the pathogenicity of *P. mononematosum* as causative agent of soft rot in yam tubers in storage, this study is the first to the current extent of our knowledge. The fungus was previously isolated from heavily molded seeds of *Amaranthus* sp. in the USA [27]. Supporting our findings of new yam's pathogen, previous investigations by Kim et al. [28] also led to the identification of *Penicillium sclerotigenum*, a novel pathogen causing yam tuber rot in Korea. It's noteworthy that the pathogenic nature of many members of the Penicillium genera is widely reported. When some are pointed out as major public health factors, affecting human and animals, some are rather ascribed to foodstuffs destruction both on-farm or in storage. In fact, several other *Penicillium* species are reported for instance as causative agents of yam tubers rot [18,29,30], or other food stuffs [31]. The host-pathogen's compatibility, as the prerequisite of successful invasion and destruction of the host by a given pathogenic agent is a result of genetic compatibility between hydrolytic enzymes and the constitutive tissues of the host. This may underlined the pathogenicity of some isolates. Meanwhile, the shift of a given isolate to pathogenic status may be due either to the absence of its specific host in a given ecosystem, or to a genetic twist orchestrated over time as a result of environmental constrains or the tropism. The missions of pathologists is therefore to track out any of these destructive agents in agricultural lands and products across the world as provide key elements which could be helpful in initiating control strategies . This study is therefore a starting point toward development of appropriate control measures to reduce the incidence of yam rots in storage in the Mbam et Kim Division and elsewhere where the disease could be found.

4. Conclusion

This investigation of the causative agents of yam rot in the main production basin of Cameroon led to the identification of seven fungi pathogens including *Fusarium solani*, *Fusarium oxysporum*, *Asperillus niger*, *Penicillum monomenatosum*, *Rhizopus oryzea*, *Talaromyces flavovirens* and *Bionectria cf. ochroleuca*. Interestingly, *P. monomenatosum* is reported for the first time as associated with yam decay. Given that the diagnosis is the first step in designing effective control measures, further investigations are currently ongoing to screen out natural products as bio-pesticides against these pathogens.

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