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Biocontrol of Cocoyam (*Colocasia esculenta*) Spoilage Fungi by *Trichoderma harzianum* collected from Rivers and Abia State, Nigeria

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ABSTRACT: Biocontrol is a technique of controlling pests, whether pest animals such as insects and mites, weeds, or pathogens affecting animals or plants by using other organisms. Hence, the objective of this paper as to investigate the biocontrol of Cocoyam (Colocasia esculenta) spoilage fungi by *Trichoderma harzianum* collected from Rivers and Abia States in Nigeria using standard microbiological methods. The results obtained show that the fungi isolated were *Aspergillus flavus*, *Aspergillus niger*, *Mucor* sp and *Penicillium* and *Trichorderma* sp. Antagonistic fungi was identified molecularly as *Trichoderma harzianum* strain A0H287. The inhibitory effect of the biological antagonist *T. harzianum* showed that it reduced the growth of *A. niger* by 50%, *Mucor* sp by 34.1%, *Penicillium* sp by 70% and *A. flavus* by 63.7%. The study showed that the biological antagonist, *Trichoderma* showed effectiveness in the reduction of the growth of majority of the pathogenic fungi and could be recommended as alternative to chemical fungicides.

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Cocoyam is a perennial monocotyledonous and herbaceous plant of the family *Araceae*. It is an important staple food in many developing countries in Africa, Asia, and the Pacific. It is one of the oldest world's food crops believed to have been first domesticated in Southeast Asia before its eventual spread to other parts of the world. The two most commonly cultivated species are Colocasia esculenta (the red type or taro) and Xanthosoma sagittifolium (the white type or tannia). In Nigeria, cocoyam is mainly cultivated for the edible corms as a source of carbohydrate to supplement yam and cassava as well as for medicinal purposes (Bartholomew *et al*, 2017). Cocoyam is believed to be consumed mostly by the low-income earners and the economically vulnerable groups. Nigeria is presently the world's leading producer of cocoyam, accounting for 35% of the world's total production. Cocoyam (Taro) is an herbaceous monocotyledonous plant of 1–2 m height. The plant consists of central corm (below the soil surface) making the leaves grow upwards, roots grown downwards, while cormels, daughter corms and runners (stolons) grow laterally. The root system is fibrous and lies mainly at a depth of up to one meter of soil (Otekunrin *et al*, 2021). The principal species of microorganism associated with cocoyam rot in Nigeria are; *Aspergillus flavus, Penicillium digitatum, Botryodiplodia theobromae and Erwinia carotovora*. These fungi are believed to be pathogenic to various cultivars of cocoyam, causing rot of several parts of Southern Nigeria. Fungi spoils the cocoyam by colonizing it by depolymerizing certain specific cell

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wall polymers such as proto-protein, the cementing substance of the produce (Agu *et al*, 2016). In Nigeria. Cocoyam has experienced declining production in recent years. Annual production in 2012 was about 15,993, which only supplied approximately 19% of local consumption. This decline is mainly attributed to common diseases that affect this crop, the most detrimental being known as "mal seco" due to its agricultural and economical repercussion. Trichoderma is a genus under fungi, which is mostly found in soil and root environments and are free-living, anamorphic, filamentous and mostly asexually reproducing (Kumar *et al*., 2023). In temperate and tropical soils, Trichoderma species form ascomycetes with green spores with hyaline and smooth septate vegetative hyphae. Studies have reported the antifungal properties of Trichoderma species cocoycam (Kumar *et al*., 2023), hence the objective of this paper as to investigate the biocontrol of Cocoyam (*Colocasia esculenta*) spoilage fungi by Trichoderma harzianum collected from Rivers and Abia States in Nigeria.

MATERIALS AND METHODS

Collection of Samples: The healthy and unhealthy cocoyam (*colocasia esculenta*) samples were collected aseptically from Rivers State and Abia state to the Microbiology laboratory for the analysis. Twenty-four (24) unhealthy tubers of cocoyam and twenty-four (24) healthy samples were collected from each states making a total of Forty-eight (48) samples. The tubers were collected in a sterile polythene bag well labeled for easy identification at the Department of Plant Science and Biotechnology, laboratory, University of Port Harcourt.

Isolation of Fungi: The method of Gwa and Abdulkadiri (2017) was adopted for the isolation of fungi from the cocoyam samples. Pieces of rotted cocoyam tubers measuring 2 × 2 mm was cut out with sterile scalpel at inter-phase between the healthy and rotten portions of the tubers. The tissue was dipped in concentration of 5% sodium hypochlorite solution for 2 minutes for surface sterilization; the sterilized sections to be inoculated was then be removed and was rinsed four times in Sterile Distilled Water (SDW). The tissue sections were placed on filter papers in the laminar air flow cabinet for 2 minutes to dry.

The pieces of the rotten cocoyam were aseptically transferred onto solidified agar medium in Petri dishes up to five pieces of the infected yam sections was inoculated on three PDA plates each. The plates were incubated for 192 hours at ambient room temperature (30 ± 5°C). Plates incubated were examined at 24 hours interval for fungal growth

Identification of the Trichoderma species and the Pathogenic Fungi: Fungi that grew from the unhealthy cocoyam pieces were sub-cultured and incubated on separate plates containing sterile acidified potato dextrose agar in order to get pure culture of the pathogenic organism. Morphological characteristics as well as microscopy of the pure cultures was made and compared with already established standard.

Microscopic Identification of Fungi: To appreciate the microscopic feature of the fungi isolated, lactophenol cotton blue was dropped on a clean glass slide, little growth of the fungus was removed with a sterile inoculating needle, and the preparation was covered with a clean cover slip and examined under the microscope with x10 magnification. Microscopic examination and morphological characteristics were noted and compared with existing authorities.

Evaluation of antagonistic activities of Trichoderma against Pathogenic Fungi: Antagonistic activities of *Trichoderma* were evaluated using dual culture method on potato dextrose agar plates. Five (5) mm diameter mycelial plugs of 5-day old fungal antagonist and pathogen were placed side by side on same Petri dish about 6 cm from each other. The antagonist and the pathogen were plated at three different times (antagonist was plated same time with the test pathogen, two days before the inoculation of the pathogen and two days after the inoculation of the pathogen). The dual and alone cultures were incubated for 192 hours at ambient room temperature (30 ± 5°C). Dishes that's only inoculated with test pathogens were used as controls. Measurement of mycelia radial growths of both the dual culture and the alone culture was carried out at 24-hour interval starting from the 72nd hour till the 192nd hour of incubation. The inhibition of the pathogen was determined according to the method of Gwa and Abdulkadir, 2017).

$$\text{PGI (\%)} = \frac{R1-R2}{R1}\ x\ 100$$

Where; PGI=Percent Growth Inhibition; R1=the radial growth of pathogen in control plate, R2=radial growth of pathogen in dual culture plate

Molecular Identification of The Fungal Isolate: Fungal genomic DNA extraction: Extraction was done using a Zymo Quick DNA Fungal/Bacterial extraction kit. A heavy growth of the pure culture of the fungal isolates was suspended in 200 microlitre of isotonic buffer into a Bashing Bead Lysis tube, DNA quality and purity were checked using NanoDrop 2000c spectrophotometer (Thermo fisher Scientific Inc.

Wilmington, Delaware, USA). Purity is measured as a ratio of absorbance at 280nm to that of 260nm.

Gel electrophoresis was performed according to the modified method of Saghai-Maroof *et al.* (1984). Sequencing was done using the Big Dye Terminator kit on on a 3510 ABI sequencer by Inqaba Biotechnological, Pretoria South Africa.

The sequencing was done at a final volume of 10ul, the components included 0.25 µlBigDye® terminator v1.1/v3.1, 2.25ul of 5 x BigDye sequencing buffer, 10uM Primer PCR primer, and 2-10ng PCR template per 100bp. The sequencing condition were as follows 32 cycles of 96°C for 10s, 55°C for 5s and 60°C for 4min. Obtained sequences were edited using the bioinformatics algorithm Trace edit, similar sequences were downloaded from the National Center for Biotechnology Information (NCBI) data base using BLASTN. These sequences were aligned using ClustalX. The evolutionary history was inferred using the Neighbor-Joining method in MEGA 6.0 (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 500 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analyzed. The evolutionary distances were computed using the Jukes-Cantor method (Jukes and Cantor 1969).

Data Analysis: The experimental design, Completely Randomized Design (CRD) will be used which will be replicated. Analysis of Variance (ANOVA) and statistical F-tests will be evaluated at $P \leq 0.05$.

RESULTS AND DISCUSSION

Macroscopic and Microscopic Characterization of the Isolates: The macroscopic and microscopic characterization of the fungi isolates is shown in Table 1. Four cocoyam pathogenic fungi were isolated in addition to an isolate of *Trichoderma.* The fungi isolated were identified based on their microscopic feature and physical colonial features. The fungi isolated *were Aspergillus flavus, Aspergillus niger, Mucor* sp and *Penicillium* and *Trichorderma* sp.

Table 1: Macroscopic and microscopic characterization of the isolates

Isolate code	Macroscopy	Microscopy	Probable fungi
T1	Ring-like, greenish woolly surface with white surrounding	Septate hyphae, conidiophores also produce chlamydospores	*Trichoderma* sp
Pf1	Floccose, greyish brown colony	Non-septate mycelium with sporgangiosphores produced in many sporangia	*Mucor* sp
Pf2	Greenish velvety surface with white, rough reverse side.	Septate hyphae with simple conidiosphores. The phialiades end having brush-like clusters.	*Penicillium sp*
Pf3	Brown-Black velvety hyphae with white surrounding and with cracked reverse	Septate hyphae with cornida arranged with cornidia like a mop-head.	*Aspergillus niger*
Pf4	Velvet green colony with the edge taped with and cream cracked reverse	Radiating cornidal heads with rough cornidiospores having a thick-walled with vesicles	Aspergillus flavus

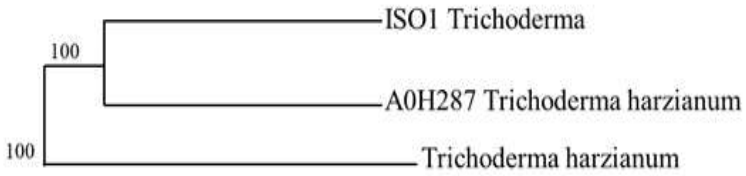

Fig 1: Phylogenetic tree showing the evolutionary distance between the fungi isolates

Molecular Identification of the Trichoderma Isolates: The agarose gel electrophoresis of the 16S rRNA gene as showed the band pair of and fungi isolates analysed. The obtained 16SrRNA sequence from the isolates produced an exact match during the megablastsearch for highly similar sequences from the NCBI non-redundant nucleotide (nr/nt) database. The 16SrRNA of the isolate ISO1 showed a percentage similarity to other species at 100%. The evolutionary distances computed using the Jukes-Cantor method were in agreement with the phylogenetic placement of the 16SrRNA of the isolate ISO1 within the *Trichorderma* genus and revealed a closed relatedness to *Trichoderma harzianum* (A0H287) than other species of *Trichoderma* and fungi (Figure 1).

Antagonistic Activity of Trichoderma harzianum on Some Pathogenic Fungi By Their Growth: The antagonistic activity of *Trichoderma harzianum* with isolated cocoyam pathogenic fungi is shown in Figure

2,3, 4 and 5 for *Aspergillus niger*, *Mucor* sp, *A. flavus* and *Penicillium* sp respectively. *Trichoderma harzianum* exhibited antagonisms against *A. niger* by an increased in growth of colony size. In the dual assay test, *Trichoderma* sp increase in size from 10mm on day 2 to 40mm on day 7 against *A. niger* which increased in the size from 23mm to 40mm on day 7. The control 1 sample which contained just the test organism, A.niger saw and increase and uninterrupted growth from 25mm on day2 to 80mm on day 7. In the dual assay of *Trichoderma harzianum* and *Mucor* sp, little or inhibition, little or no inhibition was observed as the test organism (*Mucor* sp) outgrew *T. harzianum* by increasing from 27mm on day 2 to 54mm against the growth of *Trichorderma* which increased from 24mm to 36mm on day 7. The dual assay of antagonism of *Trichoderma harzianum* and *Penicillium* sp, it was observed that *Trichoderma* grew from 23mm on day 2 to 50mm in diameter against the growth of *Penicillium* which grew from 24mm on the day 2 to 34mm on day 4 and later on through day 7, the size of growth was reduced to 24. The control sample containing only *Penicillium* recorded increased growth from 36mm on day 2 to 82 mm on day 7.

Trichoderma harzianum exhibited antagonisms against *A. flavus* by an increased in growth of colony size. In the dual assay test, *Trichoderma* sp increase in size from 26mm on day 2 to 30mm on day 7 against *A. niger* which increased in the size from 25mm to 62mm on day 7. The control 1 sample which contained just the test organism, *A.niger* saw and increase and uninterrupted growth from 25mm on day2 to 80mm on day 7. In all the fungi analyzed, it was observed that in the control plate, all the fungi grew unrestricted over a short period of time compared to when paired with the biocontrol agent, *Trichoderma harzianum* A0H287 as shown in this study

Percentage Antagonistic Effect of T. harzianum against other pathogenic fungi: The antagonistic effect of *T. harzianum* on *Mucor* sp, *Penicillium*, *Aspergillus flavus* and *Aspergillus* sp is shown in Figure 6. The inhibitory effect of the biological antagonist *T. harzianum* showed that it reduced the growth of *A. niger* by 50%, Mucor sp by 34.1%, *Penicillium* sp by 70% and *A. flavus* by 63.7%. The study showed that the biological antagonist, *Trichoderma* showed effectiveness in the reduction of the growth of majority of the pathogenic fungi, however, in the case of *Mucor* species, there was high resistance as it outgrew the *Trichoderma* sp. Cocoyam *Colocasia esculenta* is an herbaceous perennial plant which belongs to the family Araceae. Cocoyams are originally from the tropical and sub-tropical countries and studies reveal that cocoyam is among the least studied root plants (Anyanwu *et al.*, 2023). From the result obtained from this study, a total of 5 fungal isolates were obtained in from cocoyam. *Trichoderma harzianum* (antagonistic fungi) and 4 pathogenic fungi, *Mucor* sp, *Penicillium* sp, *Aspergillus flavus*, *Aspergillus niger* isolated in this study are in line with other studies; in the study of Okwu *et al.* (2020), similar fungi were identified in association with cocoyam. According to Agu *et al.* (2014; 2016), the fungi in the spoilt Cocoyam *Colocasia esculenta* were identified as *Penicillium* sp, *Aspergillus niger* and *Mucor sp*

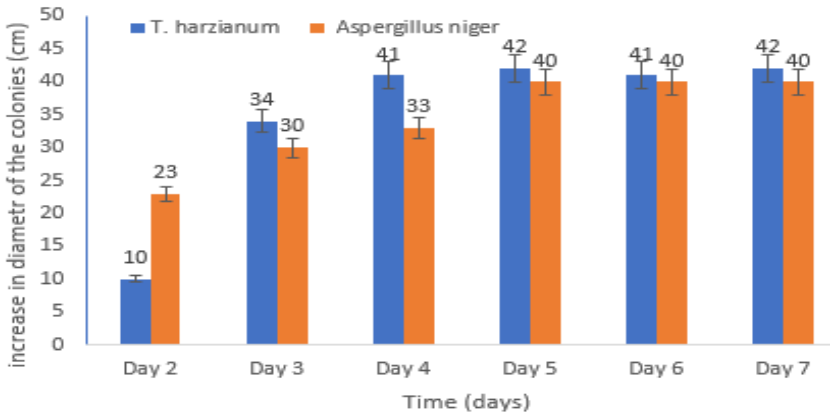

Fig 2: Growth of *Aspergillus niger* in paired cultured with *Trichoderma harzianum*

The rot due to *Aspergillus*, according to Anyanwu *et al*. (2023) is extensive resulting in complete maceration of Cocoyam *Colocasia esculenta* (Taro) tissues. The reports by Frank and Kingsley (2014) showed that the above-named organisms are actual pathogens of root and tuber crops. *Mucor* species which belonged to the group of fast-growing fungi that cause rot in Cocoyam (Anyanwu *et al.*, 2023).

Aspergillus species from spoilt Cocoyam severe rot occurrence may be due to improper storage and harvesting of Cocoyam and also due to injuries caused after harvest. The above fungi have been found to cause devastating rot blight complex (CRRBC) which is a major threat to Cocoyam production. These fungi inhabit the cocoyam through factors like; temperature and relative humidity (Agu *et al*., 2014; 2016).

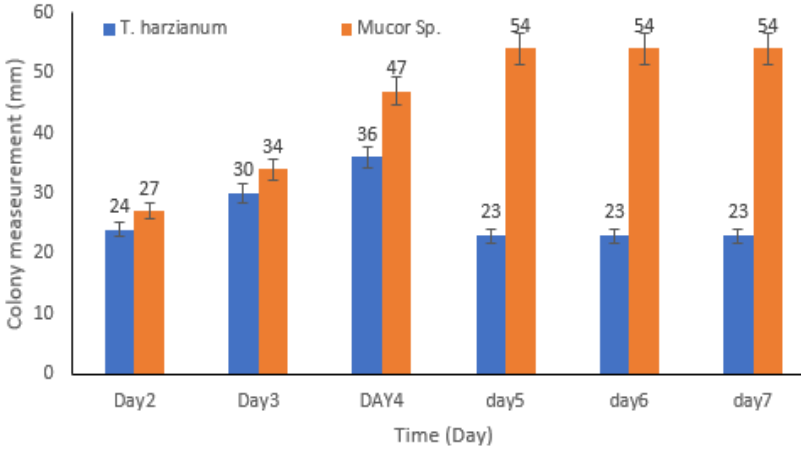

Fig 3: Growth of *Mucor sp.* in paired cultured with *Trichoderma harzianum*

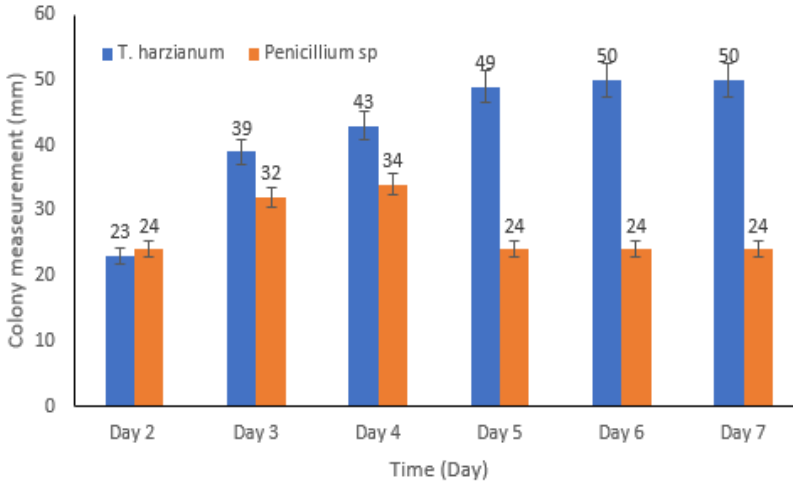

Fig 4: Growth of *Penicillium* sp in paired cultured with *Trichoderma harzianum*

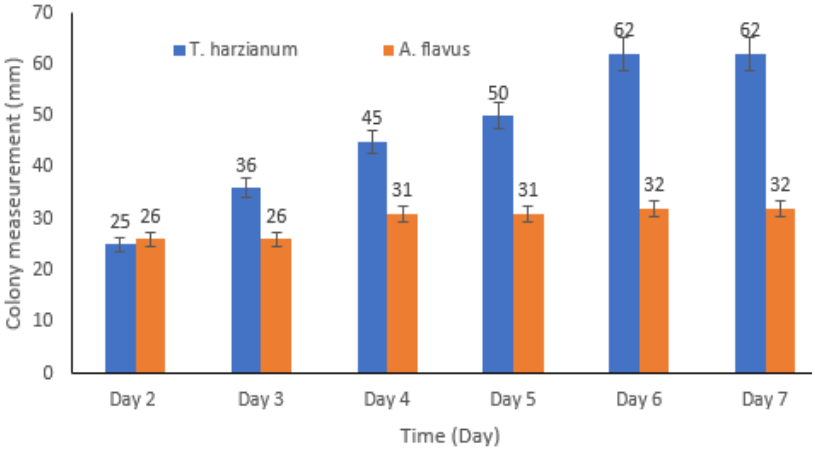

Fig 5: Growth of *A. flavus* in paired cultured with *Trichoderma harzianum*

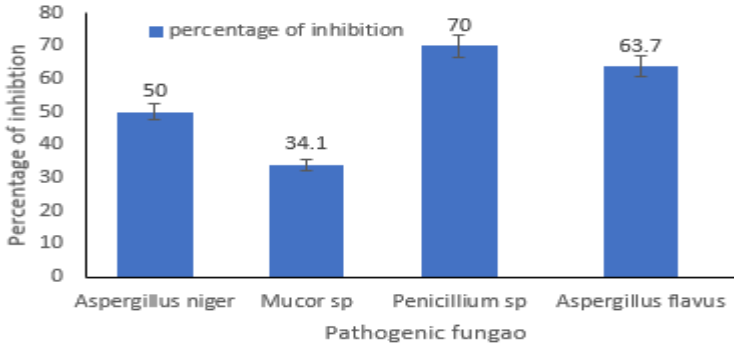

Fig 6: Percentage of inhibition of *Trichoderma* on some pathogenic fungi

One of the many biocontrol agents used to manage biotic and abiotic stress in plants are *Trichoderma* species, which predominate in the soil microflora. Due to their significant antagonistic action against a variety of phytopathogens, they can biologically control a variety of plant pathogens that cause diseases, including a range of soil and air-borne plant diseases. The ability to act as a biocontrol agent against other microbes, especially plant pathogenic fungi is a result of the production of antibiotics and mycoparasitism that affects the growth of other microbes. In addition to producing antibiotics, enzymes, volatile and non-volatile chemicals, *Trichoderma* also induces systemic resistance in plants (Kumar *et al.*, 2023). In this study, *Trichoderma harzianum* strain A0H287 was isolated and genetically identified from cocoyam samples. Previous studies have identified similar fungi have often been recognized as the most commonly proposed candidates for biocontrol. (Oskeiera *et al.*, 2015). Silva *et al.* (2019) reported that *Trichoderma harzianum* solubilizes several soil nutrients that are present in an inaccessible form and transforms them into available forms for the plants, which increases the efficiency of CO_2 and O_2 utilization in plants. Also, it is particularly helpful for the potential of *Trichoderma* to promote plant development and produce resistance through controlling the expression of genes in plants, in addition to having a direct impact on fungal plant diseases (Anyanwu *et al.*, 2023). In this study, there was an inhibition of the pathogenic fungi when paired with the biological antagonists, *T. harzianum* which is attributed to the displacement of the pathogenic fungi on the growth medium by resulting in a reduction in the percentage of growth of the pathogenic fungi. This suggest that biological control was in operation and that *T. harzianum*, acted by either producing antifungal substances or colonizing the microsites faster than naturally occurring surface pathogens (Gwa and Ekafan, 2017).

Fig 7: Dual culture of *Aspergillus flavus* and *Trichoderma harzianum*

Fig 8: Dual culture of *Aspergillus niger* and *Trichoderma harzianum*

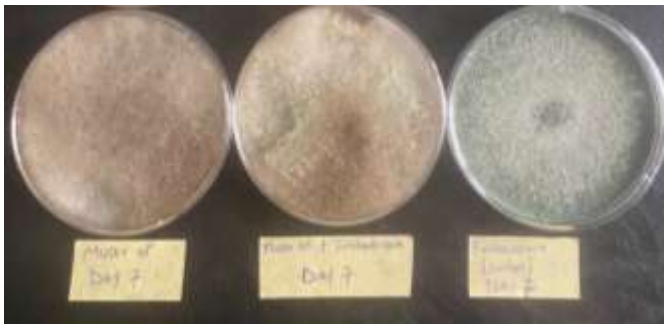

Fig 9: Dual culture of *mucor* and *Trichoderma harzianum*

Fig 10: Dual culture of *Penicillium* sp and *Trichoderma harzianum*

In this study, T. *harzianum* was used for biocontrol assay against other pathogenic fungi isolated from cocoyam and varying degree of antagonistic effect was obtained in relation to their reduction in growth for the period of 7 days. The percentage of inhibition of growth in this study showed that *T. harzianum* had the highest percentage (70%) of inhibition was observed when paired with Penicillium followed by the dual assay with *A. flavus* (63.7%) and the least percentage of inhibition was observed by *Mucor* (34.1%). This is line with previous studies which have reported varying degree of inhibition by *Trichoderma* in the biocontrol assay against other pathogenic fungi (Gwa and Ekafan, 2017). The actions of *T. harzianum* could be due to the possible role of chitinolytic and/or glucanases enzymes in bio-control by *Trichoderma* and previous studies showed that the enzymes function by breaking down the polysaccharides, chitin, and glucans that are responsible for the rigidity of fungal cell walls, thereby destroying the cell wall integrity and limiting the growth of the pathogen (Gwa and Ekafan, 2017).

Conclusion: The study showed that fungi such as *Mucor sp*, *Aspergillus niger*, *Aspergillus flavus*, *Penicillium* sp can be associated with the spoilage of cocoyam as they were isolated from spoiled cocoyam. The result of this work has shown that *T. harzianum*, has the potentials to control rot in post-harvest cocoyam as they have the ability to inhibit the growth of pathogenic fungi of cocoyam hence can provide alternative ways in reducing rot in cocoyam than in the use of chemical fungicides.

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