Antifungal Activities of Non-volatile Compound of *Trichoderma harzianum*And *Trichoderma longibrachiatum*Against RotFungi Associated with *Ananas Comosus* Linn. (Pineapple).

Comment [NY1]: Compounds
Comment [NY2]: against
Comment [NY3]: comosus

ABSTRACT

Aim: This study investigated anti-fungal properties of non-volatile compound of *Trichoderma harzianum*and *Trichoderma longibrachiatum*against rotten fungi associated with pineapple.

Comment [NY4]: compounds
Comment [NY5]: rot

Study design: This experiment was laid in a randomized complete block design.

Place and duration of study: Department of Biology, The Polytechnic, Ibadan, Nigeria, between June 2023 and September 2023

Methodology: Pineapple exhibiting symptoms of rot were purchased from Oje market within Ibadan, Nigeria. Isolation of rotten fungi was done by direct inoculating of samples showing symptoms of rot into sterilized Potato Dextrose agar (PDA). Fungal isolated were identified by using molecular techniques.The role of the isolate obtained in formation of symptoms was determined by pathogenicity test. Evaluation of non-volatile antifungal compounds of *T.harzianum* and *T.longibrachiatum* against the fungal isolates were carried out *in-vitro* at 15%, 20% and 25% concentration. All data obtained were subjected to statistical analysis.

Comment [NY6]: isolates
Comment [NY7]: isolates

Results: The mycoflora isolated were identified as*Penicilliumfuniculosum*, *Aspergillusniger* and *Meyerozymacarribica*. The isolates reproduced the same rot symptoms, while*A.niger* and *P. funiculosum*had high level of virulence with increasing incubation time. The non-volatile compounds of both biocontrol agents exert fungistatic effect on *A. niger, M. carribica*and *P. funiculosum*at 15%, 20% and 25% concentrations.

Conclusion: Therefore, ~~both~~non-volatile compounds of the bothbiological agents inhibited~~spoilage~~ growth of pathogenic fungi of pineapple *in vitro*. Thus,it could serve as a better alternative to synthetic chemical.

Comment [NY8]:
Comment [NY9]: chemicals

Key words : Pathogenicity test, Mycofloral, Fungistatic effect, Non-volatile compound

Comment [NY10]: *Mycoflora*
Comment [NY11]: *compounds*

1. INTRODUCTION

Pineapple (*Ananas comosus* L.) is a fruit with high nutritional contents, commonly grown in the tropics. It is among the most ten cultivated fruits globally and it contributes to more than 20% of the world production of tropic fruits (Liu *et al*.,2017; Chillet*et al*.,2020). Nigeria is ranked seventh among the world leading producers and the leading producer in Africa (FAO, 2019). The pineapple value chain from farm to consumption ensures food security, and creates employment while improving the rural livelihood. This identifies pineapple as a crop of economic value(Quijandria*et al*.,1997; All Africa, 2011).

However, postharvest fruit loss is estimated to account for more than 50% of the farm producelosses including pineapple in Nigeria (Moss, 2002; Zhang *et al*., 2017; Zakawa*et al*., 2019). Rot is a common problem of fleshy fruits such as pineapple. It is caused by phytopathogens such as fungi which enters the host tissue through mechanical wound and induce spoilage.The intrinsic low pH values, high sugar contents and the presence of other nutrients in pineapple are among the factors that predispose it to fungal deterioration(Onuorah *et al*., 2013). In order to limit spoilage of theproduce during storage, pineapple is treated with synthetic fungicides (Abdullah, 2011) which pose detrimental effects on human health; this necessitate the need to search for an alternative means.

In addition to being eco-friendly, biological control agents such as *Trichoderma*sppare considered a good alternative to hazardous synthetic chemicalsbecause they are host-specific, non-resistant to pathogens and effective in the short,medium and long terms. Moreover, they do not have detrimental effect on human and the environment (Carmon-Hermandez *et al*., 2019). *Trichoderma*spp shows diverse antagonistic mechanisms towards fungal phytopathogens. These could include competition, antibiosis andmycoparasitism which involves coiling of hyphae and secretion of cell wall degrading hydrolytic enzymes

Comment [NY12]: sp.
Comment [NY13]: sp.

(Bhardwajind and Kumar, 2017). Therefore, this study aimed to investigate effects of non-volatile compound of *Trichoderma* against fungal pathogens of pineapple was studied.

Comment [NY14]: Bhardwaj

2. MATERIALS AND METHODS

2.1 Sample collection

Samples of pineapples with obvious lesions were purchased and transported in sterile plastic bags to the laboratory for further analysis.

2.2 Isolation of fungi

The modified method of Odebode*et al.* (2006) was used for isolation of fungi. Tissues of pineapple were sectioned from their healthy portions to diseased portion and cut into small fragments, soaked in 5% sodium hypochlorite solution for 5minutes for surface sterilization and then rinsed thrice indistilled water. The infected tissueswere aseptically transferred to and blotted using sterile filter paper. These were aseptically inoculated onto freshly prepared plates of acidified potato dextrose agar (PDA). The plates wereincubated at 25 ± 2°C for 3-5 days. Mixed culture were subcultured by repeated culturing on freshly prepared agar until a pure culture was obtained.

2.3 Molecular characterization of Isolates

2.3.1 DNA extraction

The fungi isolates obtained and stocked in agar slants were incubated at 25 ºC for 7 days. Lysis buffer (400 mM Tris-HCL (pH 8), 60 mM EDTA- pH 8.0, 150 mM NaCl and 1% sodium dodecyl sulphate) was put in an Eppendorf tube and a small quantity of mycelium was taken from each of the slants and introduced into the tube. The tube was vortexed to disrupt the mycelia and maintained at room temperature for 10 min. Potassium acetate (150 µl) was added into the Eppendorf tube and this was followed by brief vortexing. The tube and contents were finally centrifuged at 13,000 × g for 1 min. The supernatant obtained was decanted, centrifuged again and decanted into another Eppendorf tube, to which an equal volume of isopropyl alcohol was added. After mixing by inversion, the content of the tube was centrifuged, the supernatant was discarded and the residual pellets obtained was washed with ethanol (300 µl; 70%). This was centrifuged at 10,000 rpm for 1 min followed by decantation of the supernatant. The DNA pellet was air dried and dissolved in 1×Tris- EDTA (50µl). Purified DNA (1 µl) was used in 24 µl of PCR mixture (Liu *et al*. 2000)

Comment [NY15]: fungal

2.3.2 PCR amplification

The extracted DNA was amplified by the Polymerase Chain Reaction. Each PCR mixture contained 10 µl of Red taq ready mix, 0.5 µl of each primer pair, 8 µl of analytical grade sterile water and 5 µl of genomic DNA in a total volume of 24 µl. The thermocycling program used was an initial denaturation (94 °C for 5 minutes), 30 cycles of denaturation (94 °C for 1 min), annealing (60 °C for 1 minute) and elongation (72 °C for 1 minute), then a stabilization (72 °C for 5 minutes) (Michaelsen *et al*., 2006). Amplified genetic materials were electrophoresed on 2% agarose gel in Tris acetate- EDTA buffer and the gel was stained with ethidium bromide before observation in UV detector (Okoro *et al*., 2009).

2.3.3 DNA sequencing

The sequence was determined atLaboratory of Mycology, Department of Environmental Science, University of Pavia, Pavia, Italy. The identity of the isolates were determined against known sequences in the GenBank using BLAST (Basic Local Alignment Search Tool).

2.4 Pathogenicity Test

The method of Chukwura*et al*. (2010) was adopted. Mature and fresh pineapple fruits were washed with distilled water, surface sterilized with 75% ethanol and blotted dry with filter paper. A sterile 4mm cork borer was used to bore hole in each fruit, where the isolated fungi were inoculated andthe cores were aseptically replaced to cover the hole in the fruits. The holes were sealed with petroleum jelly. All experiments including the control were placed in clean polythene bag and incubated in a humid environment at ambient temperature for ten days. All experiments were carried out in triplicates. The fruits were examined through the inoculated site for lesion development. Infected parts were aseptically transferred onto sterilized PDA to identify microorganisms responsible for the infections.

2.5 Assessment of antifungal properties of non-volatile metabolites of *Trichoderma*spp

Comment [NY16]: sp.

This was carried out according to the procedure described by Dennis and Webster (1971a). *Trichoderma* was cultivated in 250 mL conical flasks containing 100 mLpotato dextrose broth (PDB) and incubated for 10 days withperiodic shaking. The set up was filtered with Whatman no. 42 filter paper. The culture was further centrifuged at 6000rpm for 10 minutes and sterilized usinga 0.4 µm pore Millipore membrane filter. A final concentration of 15%, 20% and 25% (v/v) was obtained by mixing the culture filtrate with requisite amount of molten PDA. The fortified medium was poured into Petri dishes. A 5mm diameter mycelial plug of test pathogen was inoculated on the plates and incubated for 7 days at 28 ± 2°C.A control experiment was set up without inoculation of the antagonist. The radial mycelial growth of treatment and control were measured (to the nearest milliliter) for 7 days.

2.6 Statistical analysis

All values are mean of triplicates. Data obtained were analyzed using SPSS software subjected to analysis of variance. The means were differentiated at 5% confidence interval using Duncan's Multiple Range Test.

3. RESULTS

3.1 Isolation of fungi

Three isolates obtained from the sample were identified as *Aspergillus niger*,*Penicillium funiculosum* and *Meyerozymacarribica*.

3.2 Phylogenetic Relationships between the Isolated Fusarium moniliforme strain FM and Otherstrains Already Documented in the NCBI

It was observed that these strains showed high similarities with other fungi strains that were already documented on NCBI. It was observed that Strain tok1 is very similar to other five strains. AJ876876 was the most similar organism to strain1. Strain1 share common ancestral linage with AJ876876 and KU877217. These fungi also share common ancestor with other three fungi [MH553376, MF422165, MF422153]. It was discovered that straintok2 is mostly similar to *P.funiculosum* JX469422. However, they both share common ancestral lineage with other fungal strains. *Talaromycesstthollii* JX965246 and *Talaromycesfuniculosus* KJ728703. All these strains uniquely originated from the same ancestor which is similar to *Talaromycesfuniculosus* HG964290. The straintok3 was identified as *Meyerozymacaribbica* and phylogeny reveals that this strain is very similar to *M. Caribbica* LC422336 whom they both together share similar attributes with *M.Caribbica*LC422337 which in turn serves as an ancestor to *M. Caribbica* LC422338. Interestingly, these four strains share common striking similar ancestral lineage (0.92 pasimonial similarities) with other three *candida* strains viz: *Candida smithsoni* Ky495770 which is most similar with MG385075 but farther to MG976725.

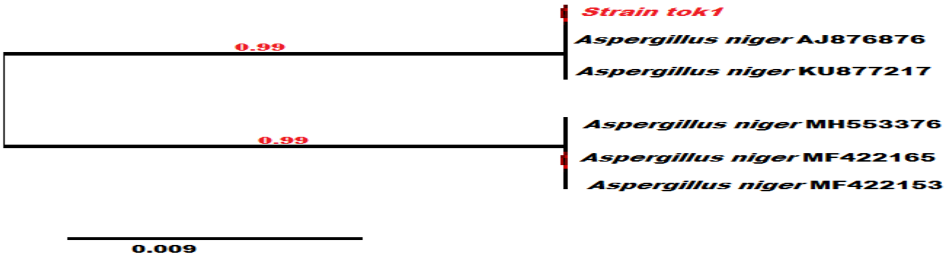

Fig. 1. Phylogenetic relationship between the isolated *Aspergillus niger* strain tok 1 and similar fungal strains already documented in National Center for Biotechnoology Information, USA (NCBI)

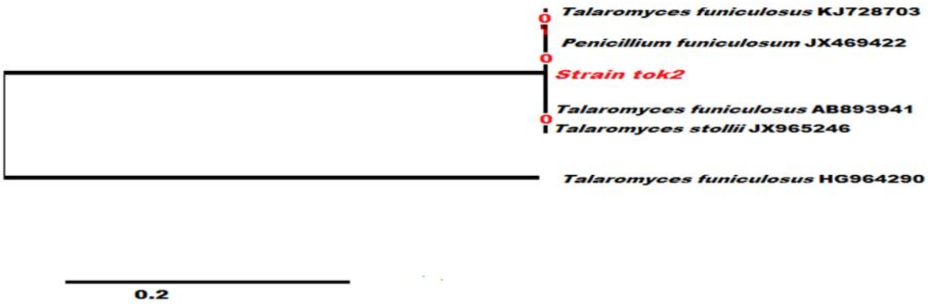

Fig. 2. Phylogenetic relationship between the isolated *Penicillium funiculosum* strain tok 2 and similar fungal strains already documented in National Center for Biotechnoology Information, USA (NCBI)

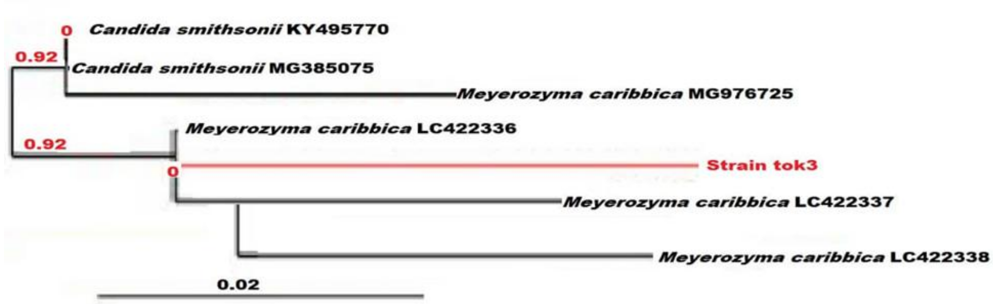

Fig. 3. Phylogenetic relationship between the isolated *Meyerozymacaribbica*ostrain FM and similar fungal strains already documented in National Center for Biotechnoology Information, USA (NCBI)

3.3Pathogenicity of pathogenic fungi causing rots in pineapple

Aspergillus niger, Meyerozymacarribica and *Penicillium funiculosum* isolated from rotten pineapples were able to incite similar rot symptoms on healthy pineapple fruits. It was observed that these pathogens caused varying levels of rot symptoms based on their varying virulence (Table 1). The symptoms observed on pineapple after re-infection with these three pathogens were more intense at days 7-8 as compared to the control. There was no visible rot symptom at days 1-3 of inoculation in the treatments, minimal between days 5 and 6 and progressing as time increased till the tenth day of incubation where virulence, measured by the length of the lesion formed was 6.07±1.79, 4.47±0.57 and 6.10±1.51 for *A. niger, M. carribica* and *P. funiculosum*respectively. *A. niger*was observed to be more virulent than *Penicillium funiculosum*but both were more virulent in causing pineapple rot than *Meyerozymacarribica*

Table 1: Virulence of the isolated pineapple rot fungi as observed from the pathogenicity test

Rot formation (cm) **Days**	***Aspergillusniger***	***Meyerozymacarribica***	***Penicilliumfuniculosum***	**Control**
Day1	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day2	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day3	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Day4	0.00±0.00	0.00±0.00	0.27±0.46	0.00±0.00
Day5	0.37±0.15	0.57±0.51	0.33±0.58	0.00±0.00
Day6	1.87±0.12	0.80±0.69	0.83±1.01	0.00±0.00
Day7	2.7±0.46	1.90±0.10	1.90±0.79	0.00±0.00
Day8	3.2667±0.40	2.27±0.38	2.53±0.74	0.00±0.00
Day9	4.90±1.31	3.73±0.46	5.27±1.45	0.00±0.00
Day10	6.07±1.79	4.47±0.57	6.10±1.51	0.00±0.00
TOTAL MEAN	**1.92^{a}**	**1.37^{d}**	**1.72^{b}**	**0.00^{e}**

Total means having the same superscript letter are not significantly different ($\alpha_{0.05}$) according to Duncan Multiple Range Test (DMRT).

3.4 Effect of Non-Volatile Compounds of *Trichoderma harzianum and Trichoderma longibrachiatum*On Pathogenic Fungi Causing Pineapple Rot

All the pathogens isolated from pineapple were susceptible to the biocontrol agents (Tables 2 and 3). *M. carribica*was the least susceptible to both *T. harzianum*and *T.longibrahiatum.*There was no inhibition of *M. carribica*at 15% concentration of *T. harzianum*inoculum while inhibition at 20% and 25% for both biocontrol agents was statistically similar. Though the bioagents were effective in the inhibition of *A. niger,* no significant difference was observed at 15% and 20% concentrations of both *T. harzianum*and *T. longibrachiatum*which improved at 25% concentration.However, *T. harzianum*significantly inhibited *A. niger.* The inhibition of the pathogen *P. funiculosum*by *T. harzianum*was most effective at 20% and 25%

Table 2: Fungistatic activities of non-volatile compound*Trichoderma harzianum* against pathogenic fungi causing pineapple rot disease

Comment [NY17]: compounds

Pathogens	Treatments	Day1	Day2	Day3	Day4	Day5	Day6	Day7	Total Mean
Aspergillus niger	15%	0.62±0.06	0.62±0.06	0.62±0.06	0.62±0.06	0.67±0.03	0.67±0.03	0.67±0.03	0.64[b]
	20%	0.55±0.17	0.57±0.20	0.57±0.20	0.58±0.19	0.70±0.13	0.70±0.13	0.70±0.13	0.62[b]
	25%	0.53±0.08	0.53±0.08	0.53±0.08	0.53±0.08	0.67±0.20	0.67±0.20	0.67±0.20	0.59[c]
	Control	0.65±0.30	1.37±0.48	2.18±0.64	3.12±1.03	4.33±0.08	4.38±0.03	4.42±0.08	2.92[a]
Meyerozymacarribica	15%	2.37±0.88	2.38±0.90	2.57±0.79	2.72±0.79	2.73±0.77	2.73±0.77	2.73±0.77	2.30[a]
	20%	1.70±0.67	1.70±0.67	1.77±0.71	1.85±0.72	1.85±0.72	1.85±0.72	1.85±0.72	1.80[b]
	25%	1.23±0.08	1.23±0.08	1.32±0.13	1.33±0.15	1.33±0.15	1.33±0.15	1.33±0.15	1.30[b]
	Control	1.30±0.17	1.40±0.13	1.75±0.10	2.78±0.41	2.87±0.46	3.07±0.81	3.17±0.75	2.63[a]
Penicillium foniculosum	15%	0.60±0.13	0.60±0.13	0.60±0.13	0.60±0.13	0.72±0.21	0.72±0.21	0.72±0.21	0.65[b]
	20%	0.43±0.06	0.43±0.06	0.58±0.08	0.58±0.08	0.63±0.10	0.63±0.10	0.63±0.10	0.56[c]
	25%	0.42±0.25	0.42±0.25	0.47±0.25	0.48±0.24	0.55±0.22	0.55±0.22	0.55±0.22	0.49[c]
	Control	0.55±0.05	1.28±0.30	3.07±0.28	3.92±0.71	4.48±0.12	4.52±0.14	4.52±0.13	3.19[a]

Values are means of three replicates, Total means having the same superscript letter are not significantly different ($\alpha_{0.05}$) according to Duncan Multiple Range Test (DMRT).

Table 3: Fungistatic activities of non-volatile compound *Trichoderma longibrachiatum* against pathogenic fungi causing pineapple rot disease

Pathogens	Treatments	Day1	Day2	Day3	Day4	Day5	Day6	Day7	Total Mean
Aspergillus niger	15%	0.68±0.18	0.78±0.25	0.82±0.28	0.87±0.30	0.92±0.38	0.92±0.38	0.92±0.38	0.84[b]
	20%	0.58±0.06	0.83±0.24	0.85±0.23	0.87±0.23	0.88±0.23	0.88±0.23	0.88±0.23	0.83[b]
	25%	0.52±0.06	0.57±0.06	0.57±0.06	0.57±0.06	0.63±0.08	0.63±0.08	0.63±0.08	0.59[c]
	Control	0.65±0.30	1.37±0.48	2.18±0.64	3.12±1.03	4.33±0.08	4.38±0.03	4.42±0.08	2.92[a]
Meyerozymacarribica	15%	2.25±0.13	2.37±0.25	2.37±0.25	2.37±0.25	2.53±0.15	2.53±0.15	2.53±0.15	2.42[b]
	20%	0.97±0.25	0.97±0.25	1.03±0.29	1.03±0.29	1.03±0.29	1.05±0.26	1.05±0.26	1.02[c]
	25%	0.88±0.23	0.90±0.26	0.90±0.26	0.90±0.26	0.95±0.23	0.97±0.21	0.97±0.21	0.92[c]
	Control	1.30±0.17	1.40±0.13	1.75±0.10	2.78±0.41	2.87±0.46	3.07±0.81	3.17±0.75	2.63[a]
Penicillium foniculosum	15%	0.72±0.13	0.78±0.12	0.88±0.10	0.88±0.10	0.92±0.08	0.92±0.08	0.92±0.08	0.86[b]
	20%	0.55±0.10	0.55±0.10	0.85±0.26	0.90±0.23	0.92±0.21	0.92±0.21	0.92±0.21	0.80[b]
	25%	0.48±0.03	0.52±0.03	0.73±0.08	0.77±0.10	0.78±0.12	0.78±0.12	0.78±0.12	0.69[bc]
	Control	0.55±0.05	1.28±0.30	3.07±0.28	3.92±0.71	4.48±0.12	4.52±0.14	4.52±0.13	3.19[a]

Values are means of three replicates, Total means having the same superscript letter are not significantly different ($\alpha_{0.05}$) according to Duncan MultipleRangeTest

Comment [NY18]: *MultipleRangeTest*

4.DISCUSSION

Analysis of the rotassociated fungi of *Ananascomosus* (pineapple) revealed the composition of the mycobiome as *Penicilliumfuniculosum*, *Aspergillusniger* and *Meyerozymacarribica*. The report of Oniah and Tawose (2018) also confirmed that *Penicillium* spp. and *Aspergillusniger*were among the organisms causing deterioration of pineapple fruits. Likewise,Ogarakue*t al*. (2016)discovered similar fungi in deteriorated pineapple fruit. *M. caribbica*has been found in many samples including in association with fruits such as sugarcane and the Brazilian fruit locally called "Camu camu" (*Myriciariadubia*) (Limtong*et al*. 2014;Matos *et al*. 2021). However, this is arguably the first time *Meyerozymacarribica*would be isolated as a causal agent of pineapple rot. It has been ascertained that fruits are rotten by phytopathogens such as fungi which infect the produce through mechanical injuries during harvesting. The isolated fungi produced similar disease symptoms when inoculated on healthy pineapple fruits. This confirms them as the causal agents of rot of the pineapple fruits, similarly, *Penicillum*spp, *Alternaria alternate* and *Aspergillus niger* have been previously reported as causative pathogens of heart rot of pomegranate fruits (Michailides*et al*. 2012; Zhang *et al*. 2012). It is also in agreement with the submission of Bastein *et al.* (2020) that the genius *Fusarium* and *Talomyces* which share a common ancestor with *Penicilliumfoniculosum* are causal agent of pineapple rot.

Different inhibitory levelwas observed among the various pathogens. Non- volatile compounds of *Trichodermaharzianum*and *Trichoderma longibrachiatum*exerted fungistatic effect on *A. niger, P. funiculosum*and *M. carribica*at concentrations of 15%, 20% and 25% in an ascending order of effectiveness.Hanan and Mohamed (2014) reported antagonistic activities of non- volatile compounds of *Trichoderma* sppagainst many phytopathogens.The fungistatic effects of non-volatile compounds of *T. harzanium*has been reported by Khaledi and Taheri (2016) where it was reported that non-volatile compoundscan decrease the mycelia growth of plant pathogens. Also, non-volatile compound of culture filtrate of *Trichoderma* strain SQR-T037 had significant effect onradial growth of *F. oxysporum. f. sp. niveum* (Waseem *et al.,* 2013).

Comment [NY19]: sp.

5. Conclusion

This study revealed the potential of using biopesticides which are biodegradable and nontoxic as a substitute to the use of expensive synthetic fungicides and other methods for plant disease control.The suppression of the fungal pathogens strongly suggests that the active components of the non-volatile compound of thebiological control agents are suitable for the control of fungi causing pineapple rot. Thus, *Trichodermaharzianum* and *Trichodermalongibrachiatum*can be used as biological control agentwhich could serve as a better alternative to the use of synthetic fungicides.

Comment [NY20]: compounds

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Comment [NY21]: Science

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