Original Research Article

Trichoderma harzianum as biocontrol agent and molecular characterisation of *Papaya ringspot virus* (PRSV) on *Cucumeropsis mannii* in Calabar, Cross River State, Nigeria

ABSTRACT

Cucumeropsis mannii is a herbaceous, perennial climbing vegetable crop in West Africa and parts of Central Africa belonging to the cucurbitacae family cultivated for it fruits and seeds. It is reported to play significant role in medicine and nutrition. Mosaic symptoms were observed on this crop during the 2021 planting season. The aim of this study was to investigate the use of *Trichoderma harzianum* as biocontrol agent against *Papaya ringspot virus* on *C. mannii* after characterising the virus from infected leave samples of *C. mannii* using RT-PCR and gene sequence analysis. The fungus *Trichoderma harzianum* was applied on the first set of polybag containing *C. mannii* followed by mechanical inoculation of the virus on leaves. Mechanical inoculation of virus was further carried out on the leaves of the second polybag containing *C. mannii.* Result obtained from Gene sequence analysis revealed 91 % nucleotide sequence identity with *Papaya ringspot virus.* This is the first report of PRSV infecting *C. mannii* in Nigeria. The result further showed that plants inoculated with a combination *Trichoderma harzianum* and virus inocula showed no symptoms while plants inoculated with virus inocula only revealed symptoms of rugosity and mottling. This results is a confirmation that *Trichoderma harzianum* is very effective against virus pathogens.

Comment [JL1]: This result indicate is better

Keywords: Cucurbitacae, Gene sequence, *Cucumeropsis mannii,* RT-PCR, *Trichoderma harzianum*

INTRODUCTION

The use of biological agent in plant diseases control has been reported to be an effective tool in the management of plant pathogenic diseases. This method is very prospective in keeping an environment clean and healthy. In this way, the ecological conscience is putting on a higher level,

starting from the most important chain in the agriculture – a producer. Biological control of pathogens is based on the use of useful alive microorganisms, which suppresses or inhibits a causing agent [5].

The most important biocontrol agents are the fungi of genus *Trichoderma*. These agents are reported to possess abilities that have been known since 1930, and now there are modern, developed technologies for using them in effective control of plant diseases. The most strains are usually present in rhizosphere. They are highly rhizosphere competitors, i.e., able to colonize and grow on roots as they develop. Colonizing them. *Trichoderma spp.* have evolved numerous mechanisms for both attack of pathogens and enhancing root and plant growth. They are in interaction with a root, soil and indirectly, with above ground parts of plants. They produce and release the lot of components, which induce the local or systemic plant resistance [14]. *Trichoderma* strains can produce extracellular enzymes and antipathogent antibiotics, but they also can be competitors of pathogens, stimulate development and induce the plant resistance [20]. The main mechanisms involved in biocontol are: antibiosis, mycoparasitism and food competition [3].

Comment [JL2]: for

The organism possesses hundreds of separate genes which act as mechanism of action. These genes offer a ”natural” crop protection and production opportunities. Several genes have been cloned from *Trichoderma spp*. that offers great promise and transgenes to produce crops resistant to plant disease [10]. Researches on the biocontrol mechanisms are interesting because of its biological properties and biotechnological applications. Biological control offers an environmentally friendly approach to the management of plant disease and can be incorporated into cultural and physical controls and limited chemical usage for an effective integrated pest management (IPM) system [20].

Papaya ringspot virus (PRSV) belongs to the genus *Potyvirus* (family *Potyviridae*), whose members have flexuous filamentous particles containing a *ca.*10 kb positive-sense single stranded RNA genome with a VPg protein at the 5’ end and a poly(A) sequence at the 3’ end [13]. This virus is transmitted by aphids in a nonpersistent manner. It can be experimentally transmitted by mechanical inoculation and some are seed-transmitted [2]. PRSV is a worldwide virus that infects mainly cucurbits and papaya with devastating consequences. Although serologically and genetically indistinguishable, two PRSV types have been described based on biological behaviour: PRSV type P (PRSV-P) infecting papaya and to a lesser extent cucurbits and PRSV type W (PRSV-W), formerly referred as WMV-1, infecting cucurbits but not papaya [19]. It is worth mentioning that PRSV-P is the major constraint for papaya but it is very uncommon in cucurbit crops, contrary to PRSV-W. However,

PRSV-P was found naturally infecting a *Cucumeropsis mannii* in Southern Nigeria [7]. [6] also reported PRSV in cucumber.

The use of antagonistic microorganisms like *T. harzianum* against plant pathogens like viruses is an alternative and contemporary way of disease control, besides the measures such as rotation of cultures, resistant varieties and use of fungicides. *T. harzianum* is one of the most effective *Trichoderma* biocontrol agents, which is commercially used for preventive protection from several plant pathogens [12]. A knowledge of this fungus related to antagonistic properties is useful for its application [9]. This research was aimed at investigating the use of *Trichoderma harzianum* as biocontrol agent against *Papaya ringspot virus* on *Cucumeropsis mannii.*

MATERIALS AND METHODS

Collection of virus samples

Papaya ringspot virus was isolated, characterised and identified by [8] in Calabar, Cross River State using the procedure stated below and thereafter maintained through mechanical inoculation on young seedlings of *Cucumeropsis mannii* in the Botanical Garden of University of Calabar, Nigeria.

RNA extraction from infected leaf samples

Total RNA was extracted from the infected leaf samples of *Cucumeropsis mannii* using the cetyltrimethylammonium bromide (CTAB) protocol as described by [1]. One hundred milligrams of each infected leaf sample was grounded in sterile mortar and pestle in 1 ml extraction buffer (100 mM Tris-HCl, pH 8.0; 1.4 M NaCl; 20 mM EDTA; 2 % CTAB) (hexadecetyltrimethylammonium bromide); and 0.4 % β- mercaptoethanol, added just before use. Each of the homogenates was poured into a new 1.5 ml Eppendorf tube. The tubes were vortexed briefly, incubated in a 60^{o}C water bath for 10 minutes and allowed to cool to room temperature. Then 0.75 ml of phenol choloform isoamyl (25:24:1) was added to each tube containing the homogenate. Each tube was then vortexed vigorously to form an emulsion and then centrifuged at the speed of 12000 rcf for 10 minutes. The supernatant was then transferred to a clean 1.5 ml tube. Three hundred of cold isopropanol was added to the supernatant to precipitate the nucleic acid (RNA) and the mixture was kept at -80^{o}C for 10 minutes. The mixture was centrifuged at 12,000 rcf for 10 min to precipitate the nucleic acid. The supernatant was discarded and the nucleic acid pellet washed in 500 µl of 70 % ethanol and centrifuged at 12,000 rcf for 5-10

minutes. The supernatant was decanted and the resultant nucleic acid pellet was air-dried at room temperature. Nucleic acid pellet was then re-suspended in 50 µl sterile distilled water and used as a template source for reversed transcriptase polymerase chain reaction (RT-PCR). Nucleic acid extracts from the leaves of healthy plants were used as negative control.

Reverse transcriptase polymerase chain reaction (RT-PCR)

Virus-specific complementary DNA (cDNA) fragments were amplified from total nucleic acid derived from the infected leaf samples by a RT-PCR method as described by [16]. RT-PCR was performed using the cylindrical inclusion (CI) primers forward 5'- TIGGIWSGGIVVIGIAARTCIAC-3', Reverse 5'-TCDATDATRTTIGACICCRTTYTIGC-3' as described by [11]. The RT-PCR reaction mixture (50 µl) consisted of I µl each of CICP 5' and CICP 3', 5x Go Taq green buffer (10.0 µl), $MgCl_2$ (3.0), dNTPs (1.0 µl), Reverse transcriptase (0.24 µl), Taq DNA polymerase (Promega) (0.24 µl), sterile distilled water (30.52 µl) and nucleic acid from infected leaf sample (1:10 dilution) (3.0 µl).

Amplifications were carried out in a GeneAmp 9700 PCR system thermalcycler (Applied Biosystem Inc., USA) using the following thermocyclic conditions; 42^{o} C for 30 min for reverse transcription, 94^{o} C for 3 min for initial denaturing, followed by 40 cycles of denaturing at 94^{o} C for 30 sec, an annealing step at 40^{o} C for 30 s, an extension at 68^{o} C for 1 min and a final extension at 72^{o} C for 10 min ended the RT-PCR reaction. The PCR reaction products were separated on 1.5 % agarose gel, subsequently stained with ethidium bromide, visualized in UV light and photographed.

Amplicon purification and sequencing

The RT-PCR amplicon for each sample was purified by adding 95 % ethanol to 40 µl of the amplicon in a new 1500 µl Ependorff tube and the solution was kept in – 80^{o} C for 10 minutes. The tube was centrifuged for 10 min and the supernatant discarded. Five hundred of 70 % ethanol was added and centrifuged at maximum speed for 5 min. The supernatant was discarded and the tube was left at room temperature to dry after which the purified cDNA was dissolved in 30 µl of sterile distil water. The product was sequenced at Bioscience Laboratory of the International Institute of Tropical Agriculture (IITA) Ibadan.

Sequence analysis

The sequence identities between the virus under study were established by comparison with known virus sequences in the GenBank available at National Center for Biotechnology Information (NCBI) using the basic local alignment search tool (BLASTn) program (http://www.ncbi.nlm.nih.gov/BLAST/).

Preparation of media for culturing *Trichoderma harzianum*

Preparation of media for culturing of *Trichoderma harzianum* was done using Potato dextrose agar (PDA). 39 grams was poured into 1000 ml conical flask. 1000ml of distilled water was added and stirred until the solution gave a paste. Non absorbent cotton wool was covered with aluminium foil and used to cork the mouth of the conical flask which was rapped again with aluminium foil up to the neck of the flask. The medium was placed in an autoclave at 121 degrees Celsius for 15 minutes. The medium was allowed to cool before removing from the autoclave and 500 milligrams of Chloraphenicol was added to the solution and allowed to cool before pouring into labelled sterile Petri dishes.

Isolation of *Trichoderma harzianum*

Soil samples collected from University of Calabar piggery farm in polyethylene bags were picked up with spatula and dropped in the plates containing PDA solution and labelled accordingly. The inoculated plates were incubated at room temperature of 27±1^{o} C and daily observations were made for emergence of fungal colonies. Colonies formed were subculture to obtain pure cultures of the isolates (Fig 1).

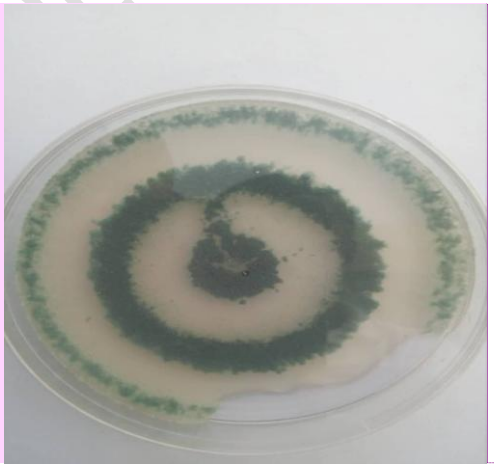

Fig 1: Colonies of *Trichoderma harzianum*

Comment [JL3]: This is result. move figures to results

Identification of *Trichoderma harzianum*

A drop of Lacto phenol in cotton blue was used to stain the slide. Sterilized inoculation needle was used to pick the spores of the fungi from culture plates and Placed on the slide containing the lacto phenol in cotton blue then covered with cover slide for observation and identification under a light microscope. (Olympus Optical Philippines) with magnification (x40). The morphological structures of the fungi were compared with those in the Atlas of Imperfect Fungi by (Barnett HL., Hunter BB. 1998). for identification.

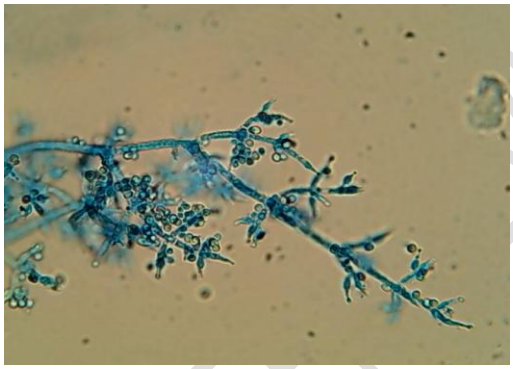

Fig 2: Photomicrograph of *Trichoderma harzianum* MAG × 400

Preparation of carrier for *Trichoderma harzianum*

Comment [JL4]: move figures to results

Preparation of carrier for *Trichoderma harzianum* species was done according to (Sivan A., Elad Y., Chet I. 1984). Three Bima bottles were used for the trial and was sterilized using sodium hypochlorite (NaOCl) solution. The bottles were rinsed in tap water, labelled accordingly and was arranged in a sterilized laboratory bench. Five grams (5g) of millet grain was used for the trial, the millet was weighed using ohaus sensitive weighing balance, and was soaked for 24 hours in 500ml of water before taken to the laboratory. The fermented millet was poured into Bima bottles and well labelled. Normal sterilization of millet grains inside the bottles was done using autoclave at the range of 121 degrees Celsius or 15 minutes. The millet grains in the sterilized Bima bottles were allowed to cool before a sterilized cork borer was used to bore and pick the fungi in petri dishes and dropped into bottles containing the substrates. Filter paper was used to cover the mouth of the bima bottles and the bored holes of the cover bottles were used to seal the mouth after inoculation. The substrates were taken immediately for inoculation.

Inoculation of *Trichoderma harzianum*

Two sets of reserved poly bags containing *Cucumeropsis mannii* seedlings, one for inoculation of *Trichoderma harzianum* and virus inocula while the second bag for the inoculation of virus inocula only, which invariably served as control. Holes were made in poly bags containing young *Cucumeropsis mannii* seedlings and the prepared millet carrier of 5 grams in Bima bottles containing spores of *Trichoderma harzianum* at 2.65 x 10^{-7}spores/ ml were inoculated into the roots of the plants and the inoculated areas were covered with soil. The virus inocula were prepared by triturating infected leaf tissue of *Cucumeropsis mannii* in pre-sterilized cold pestle and mortar in the inoculation buffer and inoculated mechanically on carborundum (600 mesh) dusted leaves of the test plant.

Inoculation of virus inocula

The second bag was inoculated with only virus inocula using the method stated above for inoculation of virus inocula

RESULTS

Nucleic acid sequencing and sequence analysis of *Papaya ringspot virus*

The result obtained after total RNA extraction, RT-PCR and gene sequence revealed fragment of the predicted size, 700 bp. Sequence analysis and comparisons using BLASTn program available at http://www.ncbi.nlm.nih.gov/BLASTn revealed 91 % nucleotide sequence identity with *Papaya ringspot virus* (Fig 3 and 4)

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ATTTGTACGGGAGATTACGTACACACCCATTTTAACGCCAGATGGAACAATAGTCAAGAAATTCAAAGGC
AATAACAGTGGCCAGCCTTCAACAGTTGTTGACAATACATTGATGGTTTTAATCACAATGTACTACGCAT
TACGTAAAGCAGGCTATGATGCAAAAGCCCAGGATGAGATGTGTGTGTTCTACATCAACGGTGATGATCT
CTGCATAGCAATTCACCCAGACCACGAGCACGTTCTTGACTCATTTTCTAACTCGTTTGCTGAGTTAGGG
CTTAAGTATGACTTCACTCAAAGGCATCGAAATAAGCAGGACTTATGGTTTATGTCACATCGAGGTGTTC
TGATCGATGACATTTACATTCCCAAACTTGAACCTGAGAGAATCGTGGCAATTCTTGAATGGGATAAGTC
CAAGCTCCCAGAGCACAGATTGGAGGCAATAACAGCAGCTTTGATAGAGTCATGGGGTTACGGAGAGCTA
ACACATCAAATTCGTAGATTCTATCAATGGGTTCTTGAACAAGCTCCGTTCAACGAATTGGCAAGACAGG
GCAGGGCTCCTTATGTCTCTGAAGTTGGTCTTAGAAGGTTGTATACAAGCAAACGTGGCTCAATGAACGA
ATTGGAAGCGTATATAGATAAATACTTTGAGTGTGAAAAAGGAGACTCACTTGAGTTGCTTGTGCACCAT
GAATCAGATGGTGCTGTGACAAAGAATCATTTTTTGTGCAGTAGCAGCAAGCACGTTTATCATCAGTCAA
AGGCTGAGGCTGTTGATGCAGGTTTCAACGATAAGCTCAAAGAGAAAGAACAAAAAGAGAAAGAGAAAAA
GAAAGAAAAAGAAAAAGACGAAGCTGGTAGCGGAAATGATGTTTCAACCAGCACGAAAACTGGAGAGAGA
GATAGAGATGTTAACGCTGGGACCAGTGGAAACTTTCACAGGTTCCAAGAATAAAGTCATTTACTG
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Fig 3: *Papaya ringspot virus* sequence

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Query  68    CTCCTTATTGAACCAACTAAACCTTTGTGTGAGAATGTCTGCAAGCAACTCAGAAGTGAT  127
             || || |||||||||||  | |||  |||||||||||||| || ||||| || ||| ||||
Sbjct  4069  CTTCTCATTGAACCAACAAGACCACTGTGTGAGAATGTATGTAAGCAGCTTAGAGGTGAA  4128

Query  128   CCATTCCACAAGAACCCAACAATTCGCATGAGAGGACTAACTTCCTTCGGTTCAACGCCA  187
             |||||  ||||||||||||||| ||  | |||||||| || ||||| || |||||||| |||
Sbjct  4129  CCATTTCACAAGAACCCAACTATCAGAATGAGAGGTCTCACTTCATTTGGTTCAACTCCA  4188

Query  188   ATTACCATCATGACTAGCGGATTTGCCTTGCATTACTTTGCGCAC--AATACAGAACAAC  245
             |||||  | ||||| || || || || ||||| |  ||| |||||  |||  ||| ||||
Sbjct  4189  ATTACAGTAATGACAAGTGGTTTCGCTTTGCACT--TTTACGCACATAATGTAGAGCAAC  4246

Query  246   TTAGGGAATTCGATTTTGTTATATTTGATGAATGCCATGTGGTTGATGCTCAGGCTATGG  305
             |    |||| ||| ||  | || ||||||||||||||| ||  |||| || ||||| ||||
Sbjct  4247  TATCTGAATACGACTTCATCATTTTTGATGAATGCCACGTAATTGACGCGCAGGCAATGG  4306

Query  306   CGTTCTACAGCTTACTCGAGGGTTATGCCATCAAAACAAAAATTCTCAAAGTGTCAGCAA  365
              ||||||  |  || |  |||   ||  ||| |||   ||  | |||||||| || ||||
Sbjct  4307  GGTTCTATTGTCTAATGAAGGAGCATAGCATTAAAGGGAAGGTCCTCAAAGTTTCTGCAA  4366

Query  366   CGCCACCTGGGCGTGAAACAGATTTCTCAACACAACATCCTGTTAAAATTGTGACTGAGA  425
             |||| |||||  | |||||||| || ||||| ||||| || || ||| | || || |||
Sbjct  4367  CGCCTCCTGGAAGGGAAACAGAATTTTCAACGCAACACCCAGTCAAATTAGTCACGGAGG  4426
Query  426   ATTCTATTAGCTTCCAACAACTGGTTGCAAGCTTTGGCACTGGTTCCACTAGTGACGTGA  485
             |    || ||||| ||||||| |||| |||| ||||||||||||  | | |||||| || |
Sbjct  4427  AAAGCATCAGCTTTCAACAATTGGTAGCAAACTTTGGCACTGGAGCGAATAGTGATGTCA  4486
Query  486   CAGCGAAAGCTGACAACATACTAGTTTATGTTGCAAGTTATAATGAAGTTGATCAATTGA  545
             |   |   ||| |||||||||| || ||||| || || || ||||||||||||||| |
Sbjct  4487  CCAAGTGCGCTAACAACATACTCGTGTATGTCGCTAGCTACAATGAAGTTGATCAACTTG  4546
Query  546   GTAAATTACTCAATGAGAGAGGATATCTAGTCACGAAAGTGGATGGGAGAACAATGAAAG  605
             | ||  |  |   ||| | ||| || || |||||||||||||||| ||||| |||||||
Sbjct  4547  GAAAGCTTTTGCTTGATAAAGGGTACCTTGTCACGAAAGTGGATGGAAGAACGATGAAAG  4606

Query  606   TTGGACGCACTGAAATAGAATCGCGAGGAACTCCACAG-AAGAAACACTTCATTGTTGCC  664
             ||||| | || || |||||| ||  ||||||    ||| |||||||| ||||||||||
Sbjct  4607  TTGGAAGAACAGAGATAGAAACGAAAGGAAC-AAGCAGCAAGAAACATTTCATTGTTGCA  4665

Query  665   ACCAACATCATCGAAAACGG  684
             |||||||| ||||| || ||
Sbjct  4666  ACCAACATTATCGAGAATGG  4685
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Fig 4: Gene sequence alignment

Use of *Trichoderma harzianum* as control agent

Trichoderma harzianum, a fungus reported to be effective as a biocontrol agent for virus pathogens was inoculated into the roots of young seedlings of *C. mannii* and immediately followed by inoculation with virus inocula on leaves of the same plant while a second pot was inoculated with virus inocula only. The results obtained after three weeks of inoculation revealed that plant inoculated with the combination of *Trichoderma harzianum* and virus inocula showed no symptoms and tested negative to RT-PCR (Fig 5a) while plant inoculated with virus inocula only revealed symptoms of rugosity and mottling and tested positive to RT-PCR (Fig 5b).

Fig 5a: C. mannii showing no symptom of infection

Fig 5b: Severe rugosity and mottling on C. Mannii

DISCUSSION

Comment [JL5]: Deepen discussion

RT-PCR and Gene Sequence Analysis

Comment [JL6]: Although important, the authors emphasized this part more, considering that biocontrol agent is the focus of the work.

The sample was detected by RT-PCR with a predicted size of 700 bp using potyvirus cylindrical inclusion (CI) primers. The gene sequence analysis revealed 91 % nucleotide sequence identity with *Papaya ringspot virus*. Detection of viruses using RT-PCR has become the most reliable method of virus diagnosis [22]. This result is consistent with the report by [8] who reported the detection of Potato virus Y using the RT-PCR procedure and gene sequence analysis. This result also confirmed the work of [7] who employed RT-PCR procedure in the detection of viruses infecting cucurbits. Though some researchers have relied on serological method like DAS-ELISA and ACP-ELISA in

plant virus diagnosis in time past however, it can be observed that recent researches have focused on the application of genetic tools in obtaining the true identify of biological entities for which this research also confirmed.

Use of *Trichoderma harzianum* as biocontrol agent

Comment [JL7]: This part requires further exploration based on the results.

Several reports have justified the use *Trichoderma harzianum* as control agent in controlling several plant pathogens. This study has revealed that leaves of C. mannii inoculated with a combination of *Trichoderma harzianum* (Control agent) and virus inocula (pathogen) showed no symptoms. Reports by [17] and [18] have revealed that Trichoderma can acts indirectly as a plant-endophyte or as a mycoparasite, through the activation of systemic plant defensive responses. Through the colonization of the roots, Trichoderma is able to activate plant defenses against the attack of pathogens, not only locally, but also systemically through responses mediated by the plant hormones salicylic acid (SA) and jasmonic acid (JA). The use of Trichoderma as a biocontrol agent requires even more studies because it effectiveness make it a sustainable alternative for the future in agricultural plant health [15].

CONCLUSION

This study was carried out to investigate the use of *Trichoderma harzianum* as biocontrol agent against *Papaya ringspot virus*. Gene sequence analysis revealed 91 % nucleotide sequence identity with *Papaya ringspot virus*. This is the first report of PRSV infecting C. mannii in Nigeria. The result further showed that *Trichoderma harzianum* is very effect in the control of virus pathogens.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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