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Trichoderma Harzianum as Biocontrol Agent and Molecular Characterisation of *Papaya ringspot virus* (PRSV) on *Cucumeropsis mannii* in Calabar, Cross River State, Nigeria

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Author's contribution

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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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.

Keywords: Cucurbitacae; gene sequence; Cucumeropsis mannii; RT-PCR; Trichoderma harzianum.

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1. 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" [1].

"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 for 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" [2]. "*Trichoderma* strains can produce extracellular enzymes and antipathogent antibiotics, but they also can be competitors of pathogens, stimulate development and induce the plant resistance" [3]. "The main mechanisms involved in biocontol are: antibiosis, mycoparasitism and food competition" [4].

"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 from Trichoderma spp. have been cloned, and transgenes to produce disease-resistant crops have been developed. " [5]. "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" [3].

"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" [6]. "This virus is transmitted by aphids in a nonpersistent manner. It can be experimentally transmitted by mechanical inoculation and some are seed-transmitted" [7]. "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" [8]. 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 [9]. [10] 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" [11]. "Knowledge of this fungus related to antagonistic properties is useful for its application" [12]. This research was aimed at investigating the use of *Trichoderma harzianum* as biocontrol agent against *Papaya ringspot virus* on *Cucumeropsis mannii.*

2. MATERIALS AND METHODS

2.1 Collection of Virus Samples

Papaya ringspot virus was isolated, characterised and identified by [13] 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.

2.2 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 [14]. 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°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°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 nucleic acid pellet was air-dried at room temperature after the supernatant was decanted. After that, the nucleic acid pellet was re-suspended in 50 µl sterile distilled water and used as a template source for reverse transcriptase polymerase chain reaction (RT-PCR). As a negative control, nucleic acid extracts from healthy plant leaves were employed.

2.3 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 [15]. RT-PCR was performed using the cylindrical inclusion (CI) primers forward 5'-TIGGIWSGGIVVIGIAARTCIAC-3', Reverse 5'-TCDATDATRTTIGACICCRTTYTIGC-3' as described by [16]. "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)" [16].

"Amplifications were performed in a GeneAmp 9700 PCR system thermalcycler (Applied Biosystem Inc., USA) using the following thermocyclic conditions; 42° C for 30 min for reverse transcription, 94° C for 3 min for initial denaturing, followed by 40 cycles of denaturing at 94° C for 30 sec, an annealing step at 40° C for 30 s, an extension at 68° C for 1 min and a final extension at 72° 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" [16].

2.4 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° 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 [17]. The product was sequenced at Bioscience Laboratory of the International Institute of Tropical Agriculture (IITA) Ibadan.

2.5 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/).

2.6 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.

2.7 Isolation of *Trichoderma harzianum*

Soil samples obtained in polyethylene bags from the University of Calabar piggery farm were picked up with a spatula and deposited into plates with PDA solution, which were then labeled. The inoculation plates were incubated at a room temperature of 27±1° C, and fungal colonies were observed on a daily basis. Colonies were created and subcultured to generate pure isolate cultures (Fig. 1).

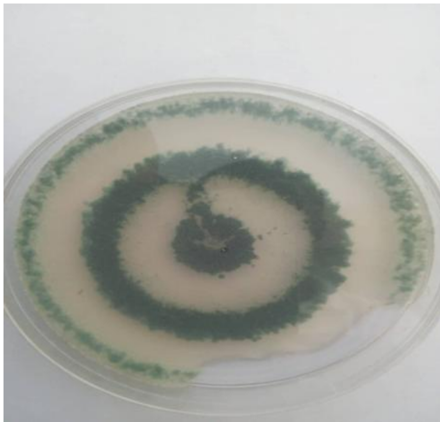

Fig. 1. Colonies of *Trichoderma harzianum*

2.8 Identification of *Trichoderma harzianum*

The slide was stained with a drop of Lacto phenol in cotton blue. The spores of the fungi were picked from culture plates using a sterile inoculation needle. Placed on a slide containing lacto phenol in cotton blue, then covered with a cover slide for analysis and identification under a light microscope (Olympus Optical Philippines) (x40). For identification, the fungi's morphological structures were compared to those in the Atlas of Imperfect Fungi (Barnett HL., Hunter BB. 1998).

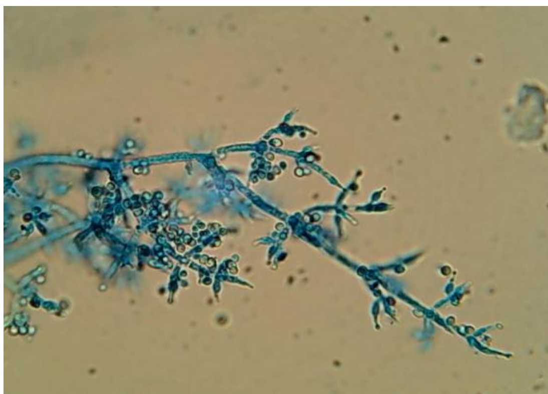

Fig. 2. Photomicrograph of *Trichoderma harzianum* MAG × 400

2.9 Preparation of Carrier for *Trichoderma harzianum*

Preparation of carrier for *Trichoderma harzianum* species was done according to [18]. “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” [18]. Then 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. The mouths of the bima bottles were covered with filter paper, and the drilled holes of the cover bottles were utilized to seal the mouths after inoculation. The substrates were removed for inoculation right away [19].

2.10 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. Inoculated mechanically on carborundum (600 mesh) dusted leaves of the test plant, the virus inocula were made by triturating infected Cucumeropsis mannii leaf tissue in a pre-sterilized cold pestle and mortar in the inoculation buffer.

2.11 Inoculation of Virus Inocula

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

3. RESULTS

3.1 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* (Figs. 3 and 4).

ATTTGTACGGGAGATTACGTACACACCCATTTTAACGCCAGATGGAACAATAGTCAAGAAATTCA
AAGGC
AATAACAGTGGCCAGCCTTCAACAGTTGTTGACAATACATTGATGGTTTTAATCACAATGTACTAC
GCAT
TACGTAAAGCAGGCTATGATGCAAAAGCCCAGGATGAGATGTGTGTGTTCTACATCAACGGTGAT
GATCT
CTGCATAGCAATTCACCCAGACCACGAGCACGTTCTTGACTCATTTTCTAACTCGTTTGCTGAGTT
AGGG
CTTAAGTATGACTTCACTCAAAGGCATCGAAATAAGCAGGACTTATGGTTTATGTCACATCGAGG
TGTTC
TGATCGATGACATTTACATTCCCAAACTTGAACCTGAGAGAATCGTGGCAATTCTTGAATGGGAT
AAGTC
CAAGCTCCCAGAGCACAGATTGGAGGCAATAACAGCAGCTTTGATAGAGTCATGGGGTTACGGA
GAGCTA
ACACATCAAATTCGTAGATTCTATCAATGGGTTCTTGAACAAGCTCCGTTCAACGAATTGGCAAGA
CAGG
GCAGGGCTCCTTATGTCTCTGAAGTTGGTCTTAGAAGGTTGTATACAAGCAAACGTGGCTCAATG
AACGA
ATTGGAAGCGTATATAGATAAATACTTTGAGTGTGAAAAAGGAGACTCACTTGAGTTGCTTGTGC
ACCAT
GAATCAGATGGTGCTGTGACAAAGAATCATTTTTTGTGCAGTAGCAGCAAGCACGTTTATCATCA
GTCAA
AGGCTGAGGCTGTTGATGCAGGTTTCAACGATAAGCTCAAAGAGAAAGAACAAAAAGAGAAAGA
GAAAAA
GAAAGAAAAAGAAAAAGACGAAGCTGGTAGCGGAAATGATGTTTCAACCAGCACGAAAACTGGA
GAGAGA
GATAGAGATGTTAACGCTGGGACCAGTGGAAACTTTCACAGGTTCCAAGAATAAAGTCATTTACT
G

Fig. 3. Nucleotide sequence of *Papaya ringspot virus* sequence

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

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

Query 188 ATTACCATCATGACTAGCGGATTTGCCTTGCATTACTTTGCGCAC--
AATACAGAACAAC 245
||||| | ||||| || || || || ||||| | ||| ||||| ||| ||| ||||

Fig. 4. Gene sequence alignment

3.2 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*

4. DISCUSSION

4.1 RT-PCR and Gene Sequence Analysis

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 [18]. This result is consistent with the report by [13] who reported the detection of Potato virus Y using the RT-PCR procedure and gene sequence analysis. This result also confirmed the work of [9] 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.

4.2 Use of *Trichoderma harzianum* as Biocontrol Agent

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 [20] and [21] 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" [22].

5. CONCLUSION

This research was carried out to investigate the use of *Trichoderma harzianum* as biocontrol agent against *Papaya ringspot virus* [23]. 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.

DISCLAIMER

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.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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