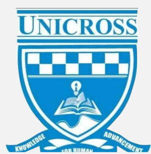

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APPLICATION OF *TRICHODERMA HARZIANUM* AS BIOCONTROL AGENT AGAINST VIRAL DISEASE OF CUCUMBER IN CROSS RIVER STATE

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Abstract

Cucumber is herbaceous, perennial climbing vegetable crop grown for it fruits belonging to the cucurbitacae family. It is reported to play significant role in medicine and nutrition. Virus infection have been reported to cause significant loses in quantity and quality of yield produced. The fungal genus *Trichoderma harzianum* is reported to play significant role as biocontrol agent in controlling virus pathogens such as fungi and viruses. Symptoms of virus infection were observed in cucumber field during the 2022 planting season. Samples were collected from 5 cucumber growing regions of Cross River State and tested against RT-PCR and gene sequence analysis was carried out. The result obtained revealed fragment of the predicted size, 700 bp. Sequence analysis using BLASTn program showed sequence identity ranging between 91 - 94 %. Viruses identified included *Morroccan watermelon mosaic virus, Papaya ringspot virus, Algerian watermelon mosaic virus, Soybean mosaic virus* and *Potato virus Y*. A combination of these viruses and *Trichoderma harzianum* were inoculated on the leaves and roots of young seedlings of cucumber respectively. The result revealed absent of symptoms while leaves inoculated with only viruses revealed symptoms of mosaic, leaf malformation and rugosity. These results have shown that the application of *Trichoderma harzianum* in virus infected fields of cucumber can go a long way in controlling the damaging effects of virus infection.

Keywords: *Trichoderma harzianum,* Viruses, Cucumber, RT-PCR, BLASTn

1.0 Introduction

Cucumber, (*Cucumis sativus L*) is a creeping plant of the gourd family (Cucurbitaceae), widely cultivated for its edible fruit. Cucumbers can help keep the body hydrated. Cucumbers have been reported to play important function in medicine and nutrition. It consumption relieves constipation, lowers the risk of cancer, keeps the body cool and hydrated, keeps the kidneys healthy, freshens breath

and natural remedy for intestinal worms (Teppner, 2004). This crop is widely cultivated in Nigeria including the South Eastern part of the country and it is subjected to more than 200 plant diseases (Lastra, 2011). Viruses rank among the most common causal agents of cucumber diseases worldwide (Provvidenti, 1996) Cucumbers have been reported to be infected by as many as 60 plant viruses worldwide and many are yet to be reported (Zitter *et al*., 1996; Lecoq *et al*., 2012).

The most commonly encountered of these viruses of are *Papaya ringspot virus*-W (PRSV-W), *Squash leaf curl virus* (SLCuV), *Soybean mosaic virus* (SMV), *Squash mosaic virus* (SqMV) and *Squash vein yellowing virus* (SqVYV). Others include *Tobacco ringspot virus* (TRSV), *Watermelon mosaic virus* (WMV), *Watermelon silver mottle virus* (WSMoV), *Alfalfa mosaic virus* (AMV), *Bean pod mottle virus* (BPMV), *Cucurbit aphid borne yellows virus* (CABYV), *Cucurbit yellow stunting disorder virus* (CYSDV), *Cucumber green mottle mosaic virus* (CGMMV), *Cucumber mosaic virus* (CMV), *Melon necrotic spot virus* (MNSV), *Zucchini yellow mosaic virus* (ZYMV), and *Zucchini green mottle mosaic virus* (ZGMMV). These viruses have emerged as the most economically significant viruses infecting cucumbers in various cucumber-growing regions of the globe (Lecoq *et al*., 2012), of these viruses CMV, SqMV, WMV-1 (now renamed WMV), WMV-2, ZYMV beside SqMV which is seedborne in melon are transmitted by beetles.

The fungal genus *Trichoderma spp*. includes about 100 described species widely distributed throughout the world due to their rapid growth, their ability to use different substrates and to tolerate the presence of different contaminants and environmental conditions (Li YY, *et al.* 2012). Its main current economic interest is based on its use as a biocontrol agent in agriculture and as a producer of enzymes in different industries (Li YY, *et al.* 2012), although in recent years its relevance in other sectors has been increasing, as a promoter of plant growth and tolerance to abiotic stresses (Poveda, 2020), biofertilizer, source of genes for use in biotechnology or mycoremediator.

In its interaction with the plant, Trichoderma mainly behaves as a root endophyte, colonizing only the outermost layers of the root, due to a plant defense response mediated by salicylic acid, which prevents the fungus from reaching the vascular bundles and behaving like a systemic pathogen (Poveda, 2020). In this way, Trichoderma is also capable of activating systemic plant defenses against the attack of pests and/or pathogens. The ways in which Trichoderma spores can be applied to crops include pre-planting applications to seed or propagation material, incorporation in the soil during seeding or transplant, watering by irrigation or applied as a root drench or dip, the greatest success colonization has been quantified by direct application to seeds and roots (Ghildiyal and Pandey A. 2015).

The comparative analysis of the genomes of *Trichoderma spp* widely used as biocontrol agents in agriculture has shown that mycoparasitism represents the ancestral way

of life of the fungal genus. The existence of a greater amount of fungal pathogens in the rhizosphere, together with the production of exudates rich in nutrients, caused Trichoderma to end up interacting with the roots, colonizing them. In this sense, their way of going evolved from mycoparasitism to a more generalist one linked to plants (Almeida *et al*., 2007). This study is therefore designed to investigate the use of *Trichoderma harzianum* as biocontrol agent against viral disease of Cucumber in Cross River.

2.0 materials and methods

2.1 Survey and Collection of virus samples

Viruses infecting cucumber were isolated during a survey in second quarter of 2022 in 5 selected cucumber growing areas in Cross River State and maintained through mechanical inoculation on young seedlings of Cucumber in the Botanical Garden of University of Cross River State, Nigeria.

2.2 RNA extraction from infected leaf samples

Total RNA was extracted from the 5 infected leaf samples of Cucumber using the cetyltrimethylammonium bromide (CTAB) protocol as described by (Abarshi *et al*, 2010). 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 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.

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 (Pappu, *et al,* 1993). RT-PCR was performed using the cylindrical inclusion (CI) primers forward 5'-TIGGIWSGGIVVIGIAARTCIAC-3', Reverse 5'-TCDATDATRTTIGACICCRTTYTIGC-3' as described by (Ha *et al*, 2008). 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° 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.

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. 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 viruses 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 will be poured into 1000 ml conical flask. 1000ml of distilled water was added and stirred until the solution gave a paste. Non absorbent cotton wool with aluminium foil will be use to cork the mouth of the conical flask and 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 collected from University of Calabar piggery farm in polyethylene bags ware collected with spatula and dropped in plates containing PDA solution and labelled accordingly. The inoculated plates was incubated at room temperature of 27±1° C and daily observations was made for emergence of fungal colonies. Colonies formed ware subculture to obtain pure cultures of the isolates

2.8 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. The morphological structures of the fungi was compared with those in the Atlas of Imperfect Fungi by (Barnett HL., Hunter BB. 1998) for identification.

2.8 Preparation of carrier for *Trichoderma harzianum*

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 sterilized using sodium hypochlorite (NaOCl) solution. The bottles were rinsed in tap water, labeled accordingly and arranged in a sterilized laboratory bench. Five grams (5g) of millet grain were used for the trial, the millet was weighed using ohaus sensitive weighing balance, and soaked for 24 hours in 500ml of water before taken to the laboratory. The fermented millet was poured into Bima bottles and well labeled. 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 was 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.

2.9 Preparation/inoculation of virus inocula

The virus inocula were prepared by triturating infected leaf tissue of cucumber in pre-sterilized cold pestle and mortar in the inoculation buffer and inoculated mechanically on carborundum (600 mesh) dusted young leaves of cucumber.

2.10 Inoculation of *Trichoderma harzianum*

Two sets of reserved poly bags containing Cucumber seedlings, one for inoculation of a combination of *Trichoderma harzianum* and virus inocula while the second bag for the inoculation of virus inocula only, which invariably will serve as control. Holes were made in poly bags containing cucumber seedlings and the prepared millet carrier of 5 grams in Bima bottles containing spores of *Trichoderma harzianum* at 2.65×10^{-7}spores/ ml were inoculated into the roots of the plants and the inoculated areas were covered with soil while the young leaves were immediately inoculated with virus inocula.

2.11 Inoculation of virus inocula

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

3.0 results

3.1 Nucleic Acid Sequencing and Sequence Analysis

The result obtained after total RNA extraction, RT-PCR and gene sequence revealed fragment of the predicted size, 700 bp. Sequence analysis using BLASTn program available at http://www.ncbi.nlm.nih.gov/BLASTn showed sequence identity ranging between 91 - 94 %. Viruses identified included

Morroccan watermelon mosaic virus, Papaya ringspot virus, Algerian watermelon mosaic virus, Soybean mosaic virus and Potato virus Y.

Table 1. Gene sequence analysis/Gene alignment with other viruses available in GenBank

S/N	Locations	Accession no	Viruses Identified	% Identity
1	Adim	KU315179.1	Morroccan watermelon mosaic virus	91
2	Okurikang	KF033100.1	Papaya ringspot virus	94
3	Akpabuyo	KU352744.1	Algerian watermelon mosaic virus	94
4	Abini	HQ396719.1	Soybean mosaic virus	93
5	Akamkpa	AF321554.1	Potato virus Y	92

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 virus infected young seedlings of cucumber and immediately followed by inoculation with virus inocula on leaves of the same plant. The results obtained after three weeks of inoculation revealed that leaves inoculated with the combination of *Trichodrma harzianum* and virus inocula showed no symptoms and tested negative to RT-PCR (Fig A and B) while leaves inoculated with virus inocula only revealed mosaic, rugosity and leaf malformation symptoms and tested positive to RT-PCR (Fig C,D,E,F and G)

A

B

Figures A and B: Cucumber leaves inoculated with the combinations of *Trichodrma harzianum* and virus inocula showing no symptoms and tested negative to RT-PCR

Figure C,D,E,F and G : Cucumber leaves leaves inoculated with virus inocula only revealed mosaic, rugosity and leaf malformation symptoms and also tested positive to RT-PCR

4.0 Discussion

4.1 RT-PCR and Gene Sequence Analysis

The detection of plant viruses using RT-PCR and gene sequence analsysis has become one of the most reliable tool in plant virus diagnosis (Usher *et al.* 2012). The result obtained from this research is consistent with the report by Eyong et al., (2022) who reported the detection of *Algerian watermelon mosaic virus, Potato virus Y* and *Papaya ringspot virus* using the RT-PCR procedure and gene sequence analysis. Eyong et al., (2020) have also employed this tool in the detection of cucurbits viruses in Cross River State. Furthermore, E Eyong et al., (2022) identified viruses infecting yams in Cross River State using RT-PCR and gene sequence analysis. Reports by Helaly et al. (2012) collaborates the use of RT-PCR and gene sequence analysis in plant virus diagnosis.

4.2 Use of *Trichoderma viride* as Biocontrol Agent for the Control of Virus Infection

Several reports have justified the use *Trichoderma harzianum* as control agent in controlling several plant pathogens. This study has revealed that leaves of cucumber inoculated with a combination of *Trichoderma harzianum* (Control agent) and virus inocula (pathogen) showed no symptoms. Reports by Poveda, (2020a) and Poveda, (2020b) 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. This results also agrees with the reports by Etim and Okon (2021) who employed the use of *Trichoderma harzianum* in the control of the viruses infecting cucumber in Cross River State

Reports have shown that 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 (Li etal 2012 and Jangir et al. 2017)

5.0 Conclusion

This study was carried out to investigate the use of *Trichoderma harzianum* as biocontrol agent against viral disease of Cucumber in Cross River State. Virus infected samples of cucumber were obtained from 5 selected cucumber production areas in Cross River State and tested against RT-PCR. The result obtained revealed fragment of the predicted size, 700 bp. Sequence analysis using BLASTn program available at http://www.ncbi.nlm.nih.gov/BLASTn showed sequence identity ranging between 91 - 94 %. Viruses identified included Morroccan watermelon mosaic virus, Papaya ringspot virus, Algerian watermelon mosaic virus, Soybean mosaic virus and Potato virus Y.

Young seedlings of cucumber were inoculated with these virus inocula and *Trichoderma harzianum*, the result revealed that *Trichoderma harzianum* was very effective in the control of virus infections as inoculated plants showed no symptoms. This research has shown that the application of *Trichoderma harzianum* in virus infected field of cucumber can go a long way in controlling the damaging effects of viruses.

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Competing interests

Authors have declared that no competing interests exist.

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