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Invitro Screening and Molecular Characterization of Trichoderma Harzianum Against Phytophthora Palmivora (Butl.,) Causing Root Rot Disease in Caricapapaya L.

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ABSTRACT

The present investigation showed Trichoderma. harzianum exhibited promising biocontrol ability against P. palmivora (Butl.) by dual culture experiment and poisoned food technique. T. harzianum was found to be the most effective antagonists against P. palmivora (Butl.). GC-MS analysis of ethyl acetate extract of the filtrate of T. harzianum revealed the presence of 17 compounds by representing 17 prominent peaks. The major phytocompound of T. harzianumwas Diethyl Phthalate (RT= 4.105 min) with 29.994 % of peak area.The ITS region gene sequence of potential biocontrol fungus obtained in this study were deposited in GenBank under the accession number KY346985. The sequence of potential biocontrol fungus showed the maximum homology with T. harzianum (GenBank Accession Number KC33021) by BLAST homology analysis. Therefore potential biocontrol fungus was further confirmed as T. harzianum by molecular analysis. The secondary structure of ITS region gene of T. harzianum showed 45 stems, 28 bulge loops and 12 hairpin like structure respectively. The free energy of ITS region gene of T. harzianum secondary structure was – 291.0 kkal/mol. The total restriction enzyme sites of ITS region gene of T. harzianum were 35. The GC and AT content of ITS region gene of T. harzianum were found to be 56 and 44% respectively. These exacting results suggested that fungicide play an important role in controlling the phytopathogenic fungi.

Key Words: Fungicides, T. harzianum, GC-MS, Biocontrol.

I. INTRODUCTION

Plant diseases caused by fungi are one of the significant destructive pathogens to economic crops of India and worldwide. The diseases produced by fungi cause a significant loss to many economic crops worldwide. The fungi generate the greatest impact in terms of reduction in crop productivity or post harvest losses and leads to a huge loss to mankind (Tapwal et al., 2011). Among the plant pathogenic fungi, P.palmivora (Butl.) causing root and stem rot are reported as the most destructive pathogens and cause extensive damage and yield losses (Al-Askar, 2012).

Trichoderma sp. are fungi that are present in nearly all soils and other diverse habitats. In soil, they frequently occur as the most prevalent culturable fungi. They are beneficial fungi that provides the most cost effective means of biocontrol preventing or controlling plant damaging pathogens. Its mechanism of action include mycoparasitism, antibiosis, competition for nutrients or space, tolerance to stress through enhanced root and plant development, solubilization and sequestration of inorganic nutrients, induced resistance and inactivation of the pathogen's enzymes (Romao-Dumaresq et al., 2012).

Papayas are highly susceptible to Phytophthora root rot. The disease occurs on papaya in the Philippines, Sri Lanka, Santo Domingo, India, Indonesia, Malaysia, Hawai'i, Mauritius, Mexico, Australia, Brazil, Spain, Taiwan, and perhaps elsewhere (Hung et al., 2015).Therefore biological control does provide an attractive and environmentally friendly option to control or suppress the development of Phytophthora diseases. Concern the above facts in mind the present investigation is justbiably designed to evaluate the antagonistic activity, culture filtrate technique, identify the bioactive compounds of potential biocontrol fungusby GC-MS analysis and study the molecular characterization of the potential biocontrol fungus.

II. MATERIALS AND METHODS

In vitro biological control of P. palmivora (Butl.) by using commonly isolated soil fungi:

Dual culture method

Colony interaction between the test pathogen P. palmivora (Butl.)and the commonly isolated soil fungi such as Aspergillus flavus, A. niger, A. repens, Cunninghamella elegans, P. nigricans, T. hamatum, T. harzianum, T. koningii, T. polysporum and T. viride were studied invitro dual culture experiments proposed by Skidmore and Dickinson (1976).

The sterilized potato dextrose agar medium supplemented with one percent streptomycin sulphate solution for preventing bacterial growth was poured into the petriplates and allowed to solidify. After solidification, colony interaction between the test pathogen

P. palmivora (Butl.) and the soil fungi were studied in vitro dual culture experiments. The test pathogen P. palmivora (Butl.) and the soil fungi such as A. flavus, A. niger, A. repens, C. elegans, P. nigricans, T. hamatum, T. harzianum, T. koningii, T. polysporum and T. viride were grown separately on PDA medium.

Then 6 mm of agar blocks cut from the actively growing margin of test pathogen and the individual species of soil fungi inoculated just opposite to each other approximately 3 cm apart on potato dextrose agar medium in petriplates. Three replicates for each set were maintained. Controls were set in single and dual inoculated culture of the fungus. The position of the colony margin on the back of the disc was recorded daily. The measurement was taken on the fifth day.

Assessments were made when the fungi has achieved an equilibrium after which there was no further alteration in the growth. Since both of the organisms were mutually inhibited, the assessment was made for both organisms.

The percentage inhibition of growth was calculated as follows.

$$\text{Percentage inhibition of growth} = \frac{r - r^1}{r} \times 100$$

r= growth of the fungus was measured from the centre of the colony towards the centre of the plate in the absence of antagonistic fungus.

r^1= growth of the fungus was measured from the centre of the colony towards the antagonistic fungus.

The colony interaction between the test pathogen and the soil fungi were assessed following the model proposed by Porter (1924) and Dickinson and Broadman (1971). Five type of interactions grade as proposed by Skidmore and Dickinson (1976) have been used.

Types are as follow

Grade 1 - Mutual intermingling growth without any microscopic sights of interaction.

Grade 2 - Intermingling growth where the fungus under observation is growing into the opposed fungus either above (or) below.

Grade 3 - Mutual intermingling growths where the growth of the fungus is ceased, and is being over grown by the opposed fungus.

Grade 4 - Slight inhibition of both the interacting fungi with a narrow demarcation line (1-2 mm).

Grade 5 - Mutual inhibition of growth at a distance of >2 mm.

Based on the antagonistic potential Trichoderma species were selected for further investigation.

Poisoned food technique

The effect of cell free culture filtrates on the growth of the pathogen was studied according to the poisoned food techniquemethod described byGrover and Moore (1962).

Preparation of culture filtrates

Agar blocks of equal size (5 mm dia) cut from the actively growing margin of the pathogenic fungus P. palmivora (Butl.)andthe antagonists T. hamatum, T. harzianum, T. koningii, T. polysporum and T. viride were inoculated separately into 250 ml conical flasks containing 100 ml of sterilized potato dextrose broth. The flasks were incubated at 27 ± 2 °C for 15 days. After 15 days of incubation the staling substances were filtered first through Whatman No.1 filter paper and then through Seitz filter. The filtrates were transferred aseptically into sterile conical flask; condensed and stored at 4°C for further use.

Assay

The culture filtrates of T. hamatum, T. harzianum, T. koningii, T. polysporum and T. viride were added separately to the cooled potato dextrose agar medium to give the concentrations of 5, 10, 15 and 20 per cent and allowed to solidify. One percent Streptomycin sulphate solution was added to the medium before pouring into petriplates for preventing bacterial growth. After solidification 5 mm agar blocks cut from the actively growing margin of the test fungus (P. palmivora (Butl.)) wasinoculated at the center of each plate. The plates were incubated at 27 ± 2°C for five days. The radial growth was measured periodically and the mean growth rate was calculated. Control was also maintained.

The percentage of inhibition of growth of pathogen was calculated as follows:

$$\text{Percentage of inhibition of growth} = \frac{\text{Growth in control} - \text{Growth in treatment}}{\text{Growth in control}} \times 100$$

sGas Chromatography and Mass Spectrometry (GC – MS) analysis of the culture filtrate ofT. harzianum:

Sample preparation

The GC –MS analysis of culture filtrate of T. harzianum was assayed by the method of Siddiquee et al. (2012). The fungus which showed the promising activity against the pathogen was cultured in a liquid potato dextrose medium at 27± 2°C, in darkness for three weeks. After incubation, the culture was filtered twice with Whatman No.1 filter paper and then through Seitz filter. To 100 ml of culture filtrate, 10 ml of ethyl acetate was added in a separation funnel (250 ml), shaken well for 3 min and the solvent and aqueous layers were separated. The ethyl acetate layer of the culture filtrate was used for further analysis.

Analysis

The GC – MS analysis was carried out using a Clarus 500 Perkin – elmer (Auto system XL) Gas Chromatograph equipped and coupled to a mass detector Turbo mass gold –Perkin Elmer Turbomass 5.1 spectrometer with an Elite – 1 (100% Dimethyl poly siloxane), 30m x 0.25 mm ID x 1µm of capillary column. The instrument was set to an initial temperature of 110 °C, and maintained at this temperature for 2 min. At the end of this period the oven temperature was rise up to 280 °C, at the rate of an increase of 5 °C/min, and maintained for 9 min. Injection port temperature was ensured as 250 °C and Helium flow rate as one ml/min. The ionization voltage was 70 eV. The samples were injected in split mode as 10:1. Mass spectral scan range was set at 45-

450 (m/z). Total GC running time was 36 min and total MS running time was also 36 min.

Identification of compounds

The time at which each constituent eluted from the GC column was termed as Retention time (RT). The eluted component was detected in the Mass detector. The spectrum of the unknown components were compared with the spectrum of the known components stored in the NIST Ver.2.1 MS data library and ascertains the name, molecular weight and structure of the components of the test materials in GC-MS study.

Molecular characterization of potential biocontrol fungus:

Isolation of Genomic DNA

The isolation of genomic DNA from filamentous fungi was studied by the method of Vazquez-Angulo et al. (2012).

Sample preparation

The potential biocontrolfungus T. harzianum wasinoculated and grown on potato dextrose broth at 28°C for 48 h. Biomass (mycelia) grown in liquid medium was collected via filtration with a Millipore apparatus, and approximately 300 mg of fungal mycelium was transferred to a 1.5-mL microtube and stored at -80°C until use.

DNA extraction buffer and solutions

The extraction buffer was 3% sodium dodecyl sulphate (w/v) containing 0.5 mM ethylenediamine tetra acetic acid, 1.0 mM NaCl, and 0.1 mM hydroxy methyl-hydrochloride (Tris-HCl, pH 8.0). Additionally, a mixture of chloroform/phenol (1:1, v/v) was also prepared.

DNA extraction procedure

Extraction buffer (0.25 mL) was added to 300 mg of each fungus mycelium and shaken vigorously for 20 sec. Then, 0.25 mL chloroform-phenol mix was slowly added and incubated at 65°C for 5 min. The mixture was centrifuged at 11,000 g at 4°C for 5 min. The supernatant (300 µL) was transferred to a new microtube, and 1-fold volume of cold absolute isopropanol was added and mixed thoroughly to precipitate total DNA at -20°C for 30 min. The mixture was then centrifuged at 11,000 g for 6 min at 4°C. The supernatant was discarded, and the pellet was resuspended in 0.03 mL diethyl pyrocarbonate-treated MiniQuantum (deionized) water and stored at -80°C for further use. Five microliters of total DNA solution was loaded onto 1% agarose gel, which was stained with ethidium bromide and electrophoresed to visualize DNA under ultraviolet light.

PCR amplification of ITS region gene

The resulting genomic DNA was used as templates to amplify the fungal ITS region gene fragment using primers NLC 2 Forward (5` GAGCTGCATTCCCAAACAACTC – 3`) and NSA 3 Reverse (5` AAACTCTGTCGTGCTGGGGATA – 3`) by Martin and Rygiewicz (2005). The PCR mixture (50 µL) contained 1 µg DNA template, 5 µL 10×Taq buffer (Fermentas), 2.5 mM $MgCl_2$, 0.2 mM dNTP mix, 0.5 µM of each primer, and 1.25 U Taq DNA Polymerase. The PCR program was carried out with 2 min at 94°C, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 56°C for 30 seconds and elongation at 72°C for 30 seconds and final elongation at 72°C for 10 min. The ITS region gene fragments of T. harzianum were identified by gel electrophoresis on 2% agarose.

Purification of PCR products

Amplified PCR products of ITS region gene of potential antagonist were eluted from the gel and then melted at 55°C with 3 volumes of 6 M guanidine thiocyanate. 20 to 30 microliters of silica mixture [1.5 % agarose gel in 1 X TAE buffer (40mM Tris – acetate, 1mM EDTA)] was added and incubation was continued for 5 mins at 55°C. After cooling on ice, the silica was pelleted, washed twice in cold 80% ethanol,

and dried in a heating block (55°C) for 1 to 2 min. The PCR fragments were eluted from the silica in 20 to 25 µl of distilled water for 3 min at 55°C.

Gene sequencing of ITS region gene

The purified PCR products were sequenced using ABI 3100 sequencer according to manufacturers' instructions (ABI PRISM 3100 Genetic Analyzer User's Manual). ITS region gene sequences of potential antagonist obtained in this study were aligned on the Bioedit software. ITS region gene sequences of potential antagonistwere deposited in Gene Bank such as National Centre for Biological Information (NCBI – http://www.ncbi.nlm,nih.gov/genebank), European Molecular Biological Laboratory (EMBL – http://www.embl.com) and DNA Data Bank of Japan (DDBJ) – http://www.ddbj.com).

Phylogenetic tree analysis

The sequences of ITS region gene of potential antagonist were compared against the sequences available from GenBank using the BLASTN program and were aligned using CLUSTAL W software developed by Higgins et al. (1992). Phylogenetic tree analysis was constructed using the Neighbour joining method (Saitou and Nei, 1987). Bootstrap analysis was done based on 1000 replications (Felsenstein, 1985). All these analysis were performed by MEGA4 package (Tamura et al., 2007).

Secondary structure prediction

The secondary structure of ITS region gene of T. harzianumwas predicted by using Genebee structure prediction software available in online (www.genebee.msu.su/service/ma2-reduced.html).

Restriction site analysis

The restriction sites in ITS region gene of T. harzianumwere analyzed by using NEB cutter program version 2.0 tools in online (www.neb.com.NEBCutter2/index.php).

III. RESULTS AND DISCUSSION

Colony interactions between P. palmivora (Butl.) and some soil fungi indual culture experiments:

The type of interactions between the pathogen P. palmivora (Butl.) and commonly isolated soil fungi such as Aspergillus flavus, A. niger, A. repens, Cunninghamella elegans, P. nigricans, Trichoderma hamatum, T. harzianum, T. koningii, T. polysporumand T. viride were observed and the types of interaction of the pathogen with soil fungi.

The maximum percentage inhibition of growth of P. palmivora (Butl.) was observedwith T. harzianum (73.85 %) followed by T. viride (72.31 %), T. koningii (70.77%), T. polysporum(69.23 %), T. hamatum (67.69 %), Aspergillus flavus (66.15 %), A. repens (64.62%), A. niger (63.08%), P. nigricans(61.54%) and Cunninghamella elegans (60.00%) in dual culture experiments (Fig.1). The results of dual culture experiments showed Trichoderma species exhibited promising activity against P. palmivora (Butl.). For this reason, Trichoderma isolates was selected for further investigation.

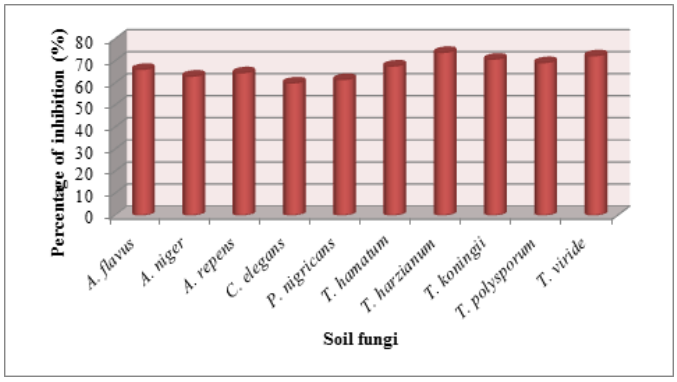

Figure 1. Percentage of inhibition of P. palmivora (Butl.) by some soil fungi

These findings are in harmony with the earlier study on the biological control of P. palmivora (Butl.) by Mpika et al. (2009) who reported twenty five Trichoderma isolates reduced the mycelial growth of P. palmivora more than 50%. Hung et al. (2015)

demonstrated antagonist activity of Chaetomium globosum CG05 expressed strongest inhibitory effects on mycelial growth of P. palmivora PHY02 causal pathogen of root rot of Pomelo (Citrus maxima).In addition, Bae et al. (2016) also reported T. atroviride and T. virens showed the strongest inhibitory activities against Phytophthora isolates.

Effect of culture filtrate of Trichoderma species on the growth of P. palmivora (Butl.)

Culture filtrate of T. hamatum, T. harzianum, T. koningii, T. polysporum and T. viride showed inhibitory effect on the growth ofP. palmivora (Butl.). The inhibitory effects of the fungi were measured as 11.94, 20.00, 15.38, 13.85 and 18.46 % at 5% concentrations, 26.15, 36.92, 33.85, 24.62 and 23.08 % at 10% concentrations, 46.15, 63.08, 44.62, 38.46 and 41.53 % at 15% and 60.00, 72.31, 63.08, 61.53 and 66.15 % at 20% concentrationsrespectively (Fig.2). The maximum inhibitory effect (72.31%) was observed at 20% concentration of the culture filtrate of T. harzianum. Results showed that fungal mycelia growth gradually decreased with increase in concentration of culture filtrate of Trichoderma species.

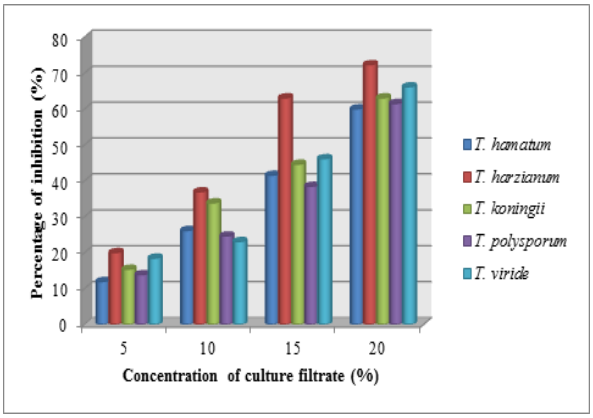

Figure 2. Percentage of inhibition of P. palmivora (Butl.) by culture filtrate of Trichoderma species

Similar work was done bySrivastav *et al.* (2011) who demonstrated potential inhibitory activity of *T. viride* and *T. harzianum* against *Phytophthora capsici* - a bell pepper pathogen. Evidently, cultural filtrate of *T. koningii* exhibited complete inhibition of *P. oryzae* and *F. oxysporum* seed borne fungi of paddy at 100% concentrationreported by Lalitha *et al.* (2012).Another study by Elshahawya *et al.* (2016) showed *T. harzianum* have antagonistic effect against *Fusarium solani, Macrophomina phaseolina* and *Sclerotinia sclerotiorum* by reducing the growth in agar assays.

Bioactive compounds identified in potential biocontrol agent of *T. harzianum* by GC-MS

The presence of chemical constituents of *T. harzianum*with their retention time, molecular formula, molecular weight and percentage of peak area are presented in table 2. A total of 17 compounds were identified from the *T. harzianum*extract. The major phytocompound of *T. harzianum*was Diethyl Phthalate (RT= 4.105 min) with 29.994 % of peak area. Vinale *et al.*, (2005)reported a large number of peptides and cyclic polypeptides antibiotics such as trichodermin, trichodermol, harzianum A and harzianolide were produced by *Trichoderma* species. Other volatile and non-volatile antifungal substances, such as diterpenes, peptaibols, butenolides, furanones, pyrones, and pyridines were also produced by *T. harzianum*. It is believed that these enzymes and antibiotics act synergistically on the host. *Trichoderma* antifungal substances are also able to arrest the hyphal growth of different fungal pathogens (Vinale *et al.*, 2008b). These findings are in analogous with the results of Siddiquee *et al.* (2012), who found that, 278 volatile compounds in *T. harzianum* strain FA1132 such as normal saturated hydrocarbons, cyclohexane, cyclopentane, fatty acids, alcohols, esters, sulfur-containing compounds, simple pyrane and benzene derivatives by using different capillary columns with nonpolar, medium polar and high polar stationary phases. The considerable potential inhibitory effect of *T. harzianum* was observed in the present study might be the presence of cumulative bioactive constituents identified by GC –MS.

Table 2. Bioactive compounds identified in *T. harzianum* by GC-MS

S. No.	RT	Name of the compound	Molecular Formulae	MW	Peak Area %
1.	4.105	Diethyl Phthalate	$C_{12}H_{14}O_4$	222	29.994
2.	4.255	1-Iodo-2-methylundecane	$C_{12}H_{25}I$	296	8.881
3.	6.453	Phenyl salicylate	$C_{13}H_{10}O_3$	214	7.892
4.	7.083	1-Iodo-2-methylundecane	$C_{12}H_{25}I$	296	0.032
5.	7.510	3-Eicosene, (E)-	$C_{20}H_{40}$	280	5.833
6.	10.192	Phthalic acid, isobutyl octadecyl ester	$C_{30}H_{50}O_4$	474	0.549
7.	10.550	1-Iodo-2-methylundecane	$C_{12}H_{25}I$	296	0.912
8.	11.097	Sulfurous acid, butyl dodecyl ester	$C_{16}H_{34}O_3S$	306	2.862
9.	12.550	9,10-Anthracenedione, 2-methyl-	$C_{15}H_{10}O_2$	222	2.214
10.	13.753	Hexadecane, 1,1-bis(dodecyloxy)-	$C_{40}H_{82}O_2$	594	0.196
11.	14.329	Allopregnane-7.alpha.,11.alpha.-diol-3,20-dione	$C_{21}H_{32}O_4$	348	0.050
12.	17.438	4,25-Secoobscurinervan-4-one, O-acetyl-22-ethyl-15,16-dimethoxy	$C_{27}H_{36}N_2O_6$	484	0.171
13.	17.900	Heptacosane, 1-chloro-	$C_{27}H_{55}Cl$	414	0.419
14.	19.820	Phthalic acid, 2-ethylhexyl tetradecyl ester	$C_{30}H_{50}O_4$	474	1.005
15.	20.312	2-methyloctacosane	$C_{29}H_{60}$	408	0.374
16.	21.659	Sulfurous acid, hexyl pentadecyl ester	$C_{21}H_{44}O_3S$	376	1.132
17.	22.434	2-Myristynoyl pantetheine	$C_{25}H_{44}N_2O_5S$	484	0.449

RT – Retention Time, MW – Molecular Weight

Molecular characterization of potential biocontrol fungus *T. harzianum*

The molecular characterization of potential biocontrol fungus *T. harzianum* was done by ITS region gene sequencing analysis. The amplification of the ITS region gene was confirmed by agarose gel electrophoresis. The PCR product was gel eluted and sequenced. The ITS region gene sequence of potential biocontrol fungus obtained in this study were deposited in GenBank under the accession number KY346985 (Fig. 4). BLAST homology analysis was also carried out to compare with other ITS region gene partial and complete sequences available in the GenBank of NCBI and it revealed that the sequence of potential biocontrol fungus *T. harzianum* showed the homology (93%) with *T. harzianum* (GenBank Accession Number KC330218). Therefore, the potential biocontrol fungus was further confirmed as *T. harzianum* by molecular analysis.

Molecular techniques exhibit high sensitivity and specificity for identifying microorganisms and can be used for classifying microbial strains at diverse hierarchical taxonomic levels (Sette *et al.*, 2006). Moreover Siddiquee *et al.* (2007) reported thirty-six isolates were positively identified as *T. harzianum* (32

strains), *T. virens* (3 strains) and *T. longibrachiatum* (1 strain) by ITS region sequences analysis.

12/21/2016 Trichoderma harzianum isolate soil sample small subunit ribosomal RNA - Nucleotide - NCBI

Nucleotide

GenBank

Trichoderma harzianum isolate soil sample small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence

GenBank: KY346985.1

FASTA Graphics

Go to:

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Figure 4. ITS region gene sequence of *T. harzianum*

Evolutionary relationships of potential biocontrol fungus *T. harzianum*

The evolutionary relationships of 16 taxa were inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed. The topology of the NJ tree inferred from the whole dataset was showed in Figure 5. The overall tree topology suggested that the tree was divided into 2 main clades namely A, B. The clade A

had 7 taxa and clade B with 9 taxa including the test fungus *T. harzianum*. The test strain *T. harzianum* was shared with *T. harzianum* (GenBank Accession Number KC33021) in clade B.

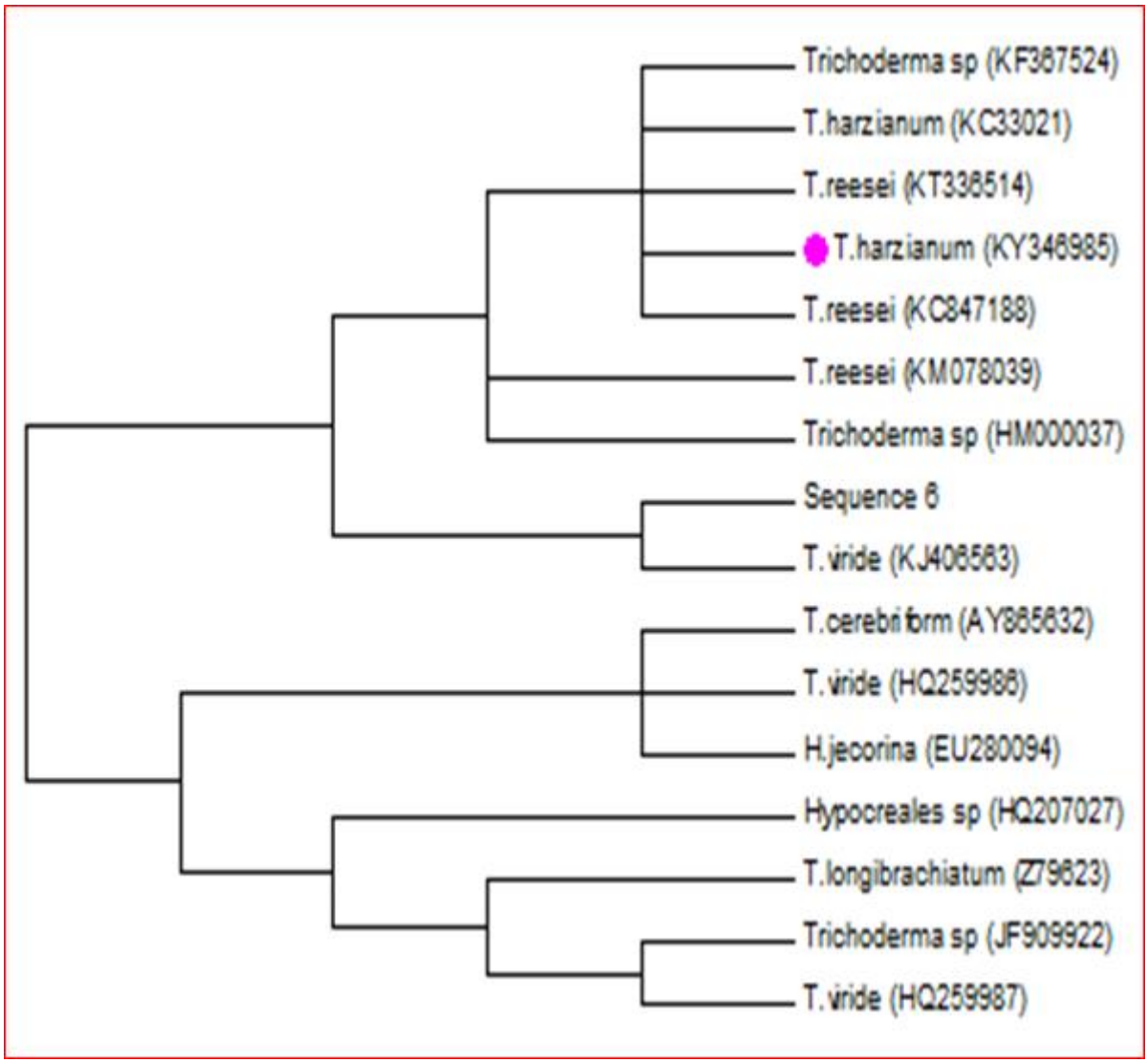

Figure 5. Phylogenetic treeanalysis of *T. harzianum* - Evolutionary relationships of 16 taxa

Kubicek *et al*. (2003) demonstrated that seventy-eight isolates of *Trichoderma,* 37 strains were positively identified as *T. harzianum* by molecular phylogenetic analyses. These reports demonstrated molecular techniques indicating interrelations among species and, while combined with phenotypic characters, can lead to a reliable taxonomy that is reflective of phylogenetic relationships. Likewise Senthilkumar *et al*. (2011) accounted the isolate *T. harzianum* has been found closely related to already existing species of *Hypocera tixii* at 99% of similarity.

Secondary structure prediction

The secondary structure prediction of *T. harzianum* showed 45 stems, 28 bulge loops and 12 hairpin loops in their structure (Fig. 6). The free energy of ITS region gene of *T. harzianum* secondary structure is -291.0 kkal/mol.

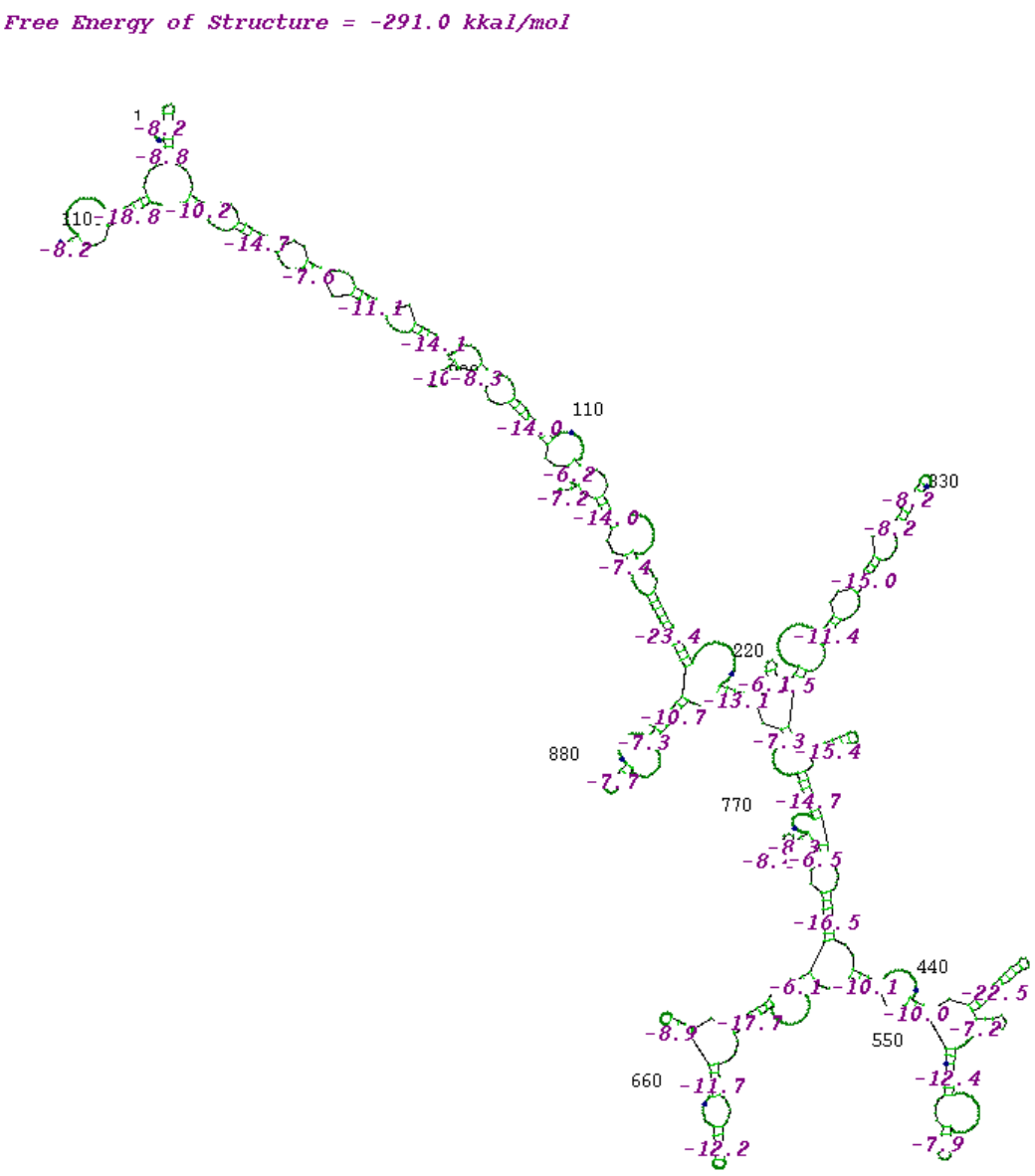

Figure 6. Secondary structure of ITS region gene of *T. harzianum*

Previously Senthilkumar et al., (2011) reported the secondary structure of T. harzianum 18S rDNA (GU646678) showed 25 stems, 24 bulge loops and 14 hairpin loops in their structure. Likewise Hamed et al., (2015) accounted the RNA secondary structure was predicted for 18S rRNA of SRBP_ZSHSG1. It showed that the free energy of structure is -150.3 kkal/mol, threshold energy is -4.0 with cluster factor, conserved factor 2 and compensated factor 4 and conservatively is 0.8.

Restriction sites analysis

The restriction sites of ITS region gene of T. harzianum were shown in Fig. 7. A large number of restriction sites were found in potential antagonist. The total restriction enzyme sites of T. harzianum is 35. However, the cleavage sites and the nature of restriction enzymes differed from one another. The GC and AT content of ITS region gene of T. harzianum was found to be 56 and 44% respectively using NEB Cutter Programme V 2.0 in www.neb.com/nebcutter2/index.php .

Similarly, Fahmiet al., (2016) also showed the distribution of nucleotides as well as the guanosine + cytosine (G+C) content in ITS1+5.8S+ITS2 complete sequences. A total length of ITS1-5.8S-ITS2 sequence region ranged from 546 bp to 1028 bp in all accessions. While, the G+C content ranged from 24.2% to 68.8%.In recent times, gene sequences of T. harzianum and their phylogenetic relationships, secondary structures and restriction enzyme sites have been reported by Gherbawy et al., (2014).

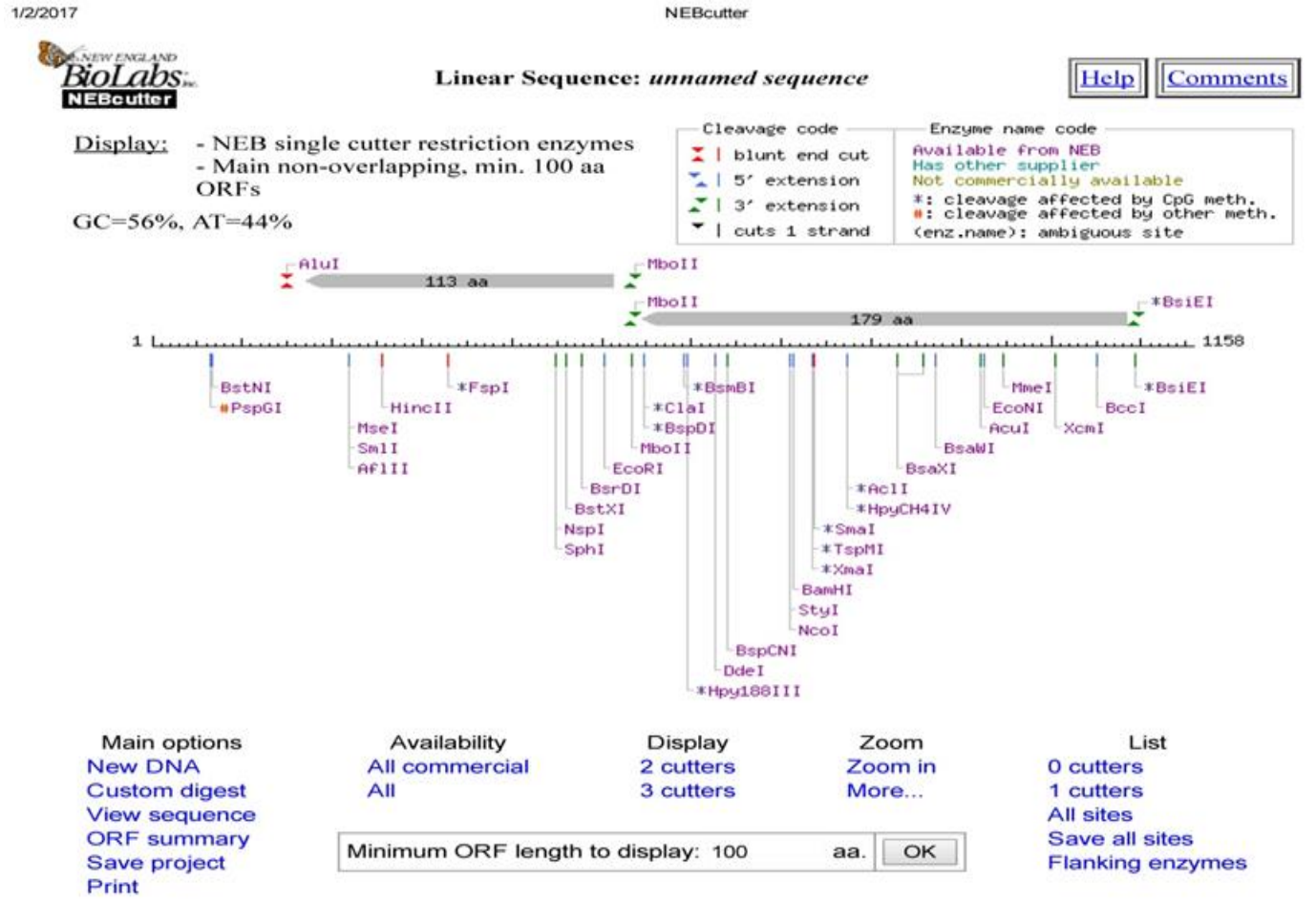

Figure 7. Restriction site analysis and GC and AT content of ITS region gene of *T. harzianum*

IV. CONCLUSION

Conclusion of the present research work, invitro screening of Trichoderma species has given encouraging results, indicating their potential use in the management ofPhytophthora root rot disease in Papaya plant caused by P. palmivora (Butl.). Trichoderma species are prospective source for biocontrol agents and its play an important role in soil fertility and promoting plant growth.

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