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Identification of Three Biocontrol Genes (ech-42,chit-HAR3 and Xyn2) Involved in Fungal/ Bacterial Cell Wall Degradation in *T.harzianum* (*Th* Azad/6796)

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The members of the genus *Trichoderma* are widely used as bioagent for the control of phytopathogenic fungi in agriculture sector. The members of this genus are reproducing asexually by the formation of cyanide and chlamydospore, in wild habitats they reproduce by formation of ascospores. We all know that all living organisms are made up genes that code for a particular function. Similarly, in *Trichoderma* some genes are responsible for the secretion of these CWDEs. These genes which aid in the biocontrol action are called biocontrol genes. These bacterial genes code for a particular enzyme and protein that degrade the pathogen cell wall. These biocontrol genes can be isolated and cloned for large scale commercial production. It has also been found that some of the genes of *Trichoderma* are also helpful in the abiotic and biotic stress. The mechanisms which are employed by *Trichoderma* for the phytopathogenic action are generally included antibiosis, mycoparasitism competition for nutrients etc. In the present investigation three biocontol genes (ech-42,chit-HAR3 and Xyn2) were identified in *Trichoderma harzianum*.

Keywords: *Trichoderma,* Biocontrol genes, CWDEs, Sequence**.**

Plant Pathogens cause major disease in agriculture crops. *Trichoderma* is widely used for the control of these plant pathogens. *Trichoderma* species are well known for the production of Cell wall degrading enzymes (CWDEs), theses CWDEs these can be commercialized on large scale. We all know that living organisms are made up of genes that code for a specific function these genes which play role in biocontrol process are known as biocontrol genes. (Pandey *et al*, 2014 A b,Srivastava *et al*, 2015). When these genes come in contact with phytopathogens send some signals. These signal triggers the secretion of proteins and enzymes that degrade the phytopathogens. These biocontrol genes can be cloned in large quantities' for commercial production (Massart and Jijakli, 2007). Resistance to abiotic stresses such as heat, drought, salt etc. can be provided by the use of some genes of *Trichoderma* (Kuc, 2001). Antibiosis, mycoparasitism are the main biocontrol mechanisms that are employed by *Trichoderma* against phytopathogens. *Trichoderma* is widely used as biocontrol agent due to these characters. chitinase, xylanase. stress tolerant genes are the major kind of genes whose isolation and cloning is easy. Cell wall degradation, hyphal growth retardation is the major role of these genes. Glycosidic bonds are breakdown by chitinase enzymes. D-glucose to D-glucono-1, 5-lactone and hydrogen peroxide catalyses by antifungal enzyme

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glucose oxidase. Hemicellulose can be break by xylanase which is major component of plant cell walls. *Th*- Chit, a gene isolated from *Trichoderma harzianum* have found to possess antifungal chitinase activity against transgenic tomato plant. *Tri5* is a gene isolated from *T. brevocompactum* is responsible for the synthesis trichodiene, the production of this compound regulate the synthesis of trichodiene in which have found to possess antifungal activity against *S.cerevisiae, Candida albicans, C. glabrata, C. tropicalis* and *Aspergillus fumigates*. A gene called *erg1* was isolated from *T. harzianum* was cloned, characterized and have been found to possess squalene peroxidase activivty which responsible for the synthesis of ergosterol and providing resistance to terbinafine (Cardoza *et al*., 2006). From *T. hamatum* a monooxygenase gene was isolated cloned and characterize, this gene has antifungal activity against *S. sclerotiorum, Sclerotinia minor,* and *Sclerotium cepivorum*. From 5 isolates of *T. harzianum* namely (*T 30, 31, 32, 57 and 78*) genes were isolated that are responsible for encoding N-acetyl-â-D-glucosaminidase (*exc1* and *exc2*), chitinase (*chit42* and *chit33*), protease (*prb1*) and â-glucanase (*bgn13.1*). These genes are effective against *Fusarium oxysporum*.A gene responsible for the expression of â-1,4-endoglucanase was isolated from *T. longibrachiatum*. This gene was found to be effective against the *P. ultimum*, causing damping off of cucumber. A gene named egl1 responsible for endoglucanase activity was also isolated from *T. longibrachiatum* (Migheli *et al*., 1998).

From *T. harzianum* CECT 2413, a gene nemed , *qid74* was obtained this gene is found to play a significant role against *R. solani*. From *T. aueroviride* a gene named *Taabc2* was isolated this has function in ATP binding cassette (ABC) transporter in cell membrane pump that helps in the mycoparasitic activity. The antagonist activity of this was tested against pathogens such as *R. solani, B. cinerea, and P. ultimum* was done by dual culture plate assay with *T. atroviride* wild and mutant type strains (Ruocco *et al*., 2009). A gene encoding adenylate cyclase *tac1,* was isolated from *T. virens,* This gene is found to play a role against *R. solani* and *P. ultimum* (Mukherjee *et al*., 2007). From *T. virens* glutathione transferase gene *TvGST* was cloned. This gene is responsible for the cadmium resistance (Dixit *et al*., 2011). A gene *hsp70* isolated from *T. herzianum T34* has resistance against heat and salt (Mantero Barrientos *et al*., (2008). A gene named *Thkel1*, isolated from *Trichoderma harzianum* is shown to enhance glucosidae activity, and has found to have a profound effect on salt and osmotic stress (Hermosa *et al*., 2011).

MATERIALS AND METHODS

Trichoderma species used in this study was isolated from rhizosperic soils collected from the different locations of U.P. and maintained on potato dextrose agar (PDA) (Himedia, USA) at 28±2°C for 5 days. Ten *Trichoderma* species were isolated from rhizosphere samples according to soil dilution plate method described by Kucuk and Kivanc (2003) with some modification. One ml of each appropriate dilution (10^{-3} to 10^{-5}) was pipetted in petri dishes, then the sterilized and cooled at 45°C Rose Bengal Agar medium (RBA) was poured and left to solidified. All plates were incubated at 28°C for 7 days. The culture plates were examined daily and individual colonies were isolated and purified, then transferred to fresh potato dextrose Agar medium (PDA). Distinct morphological characteristics were observed for identification and the plates were stored at 4°C for further experiments.

The nucleotide sequences of all the isolated strains submitted to NCBI (http://www.ncbi.nlm.nih.gov/) are retrieved and the genes responsible for cell wall degradation are identified and retrieved such as chi1, chit-HAR3, ech42 and xyn2 coding for chitinase, endochitinase, and xylanase respectively. The gene sequences are then analyzed using bioinformatics tools and methods (Shahid *et al*, 2013 a b).

Identification of genes involved in fungal/ bacterial cell wall degradations

The following steps have been undertaken for the gene-identification:

Similarity search using BLAST

All the nucleotide sequences of the 7 isolated strains of *Trichoderma* sp. are searched in BLASTn tool for finding similar templates. All the seven strains showed significant similarity to the other strains of *Trichoderma*.

Sequence Analysis

The nucleotide sequences of the identified genes are searched for promoters using NNPP (Neural Network Promoter Prediction) program. Promoter prediction is an important step in sequence analysis as it helps us to identify the coding regions and their location in the complete genomic sequence. The positions and patterns of the predicted promoters for the identified genes have been determined.

Gene-finding

Searching the query sequences against EST database and finding ORFs is the principle on which gene finding is based in this report. Variants of BLAST such as BLASTx, BLASTp and BLASTn (against EST database) are used for finding the coding regions and conserved domains present in the gene sequences. GENSCAN is used for peptide prediction and the peptides thus predicted are searched through BLASTx program for validation.

RESULTS

Isolate was identified up to species level based on phenotypic characters like colony colour and growth; size and shape of conidiophore, phialides and conidia using the available literature (Bisset 1991a,1991b and Samuels *et al.*1998) and confirmed by SEM analysis (Fig. 1), as well as molecular identification by ITS marker, sequences deposited to NCBI GenBank (Acc. No.-KC800922) and re-confirmation by ITCC, New Delhi (Acc. No.-ITCC-6796). Finally, potential and effective bio-agent *T. harzianum* (*Th* Azad/6796) was submitted to microbial data bank at NBAIM, Mau (Acc.No.-F-03109).

Identification of genes involved in fungal/ bacterial cell wall degradations

Plant genes respond to pathogen by their immune response system. this immune response does not necessarily require any stimulation. it has

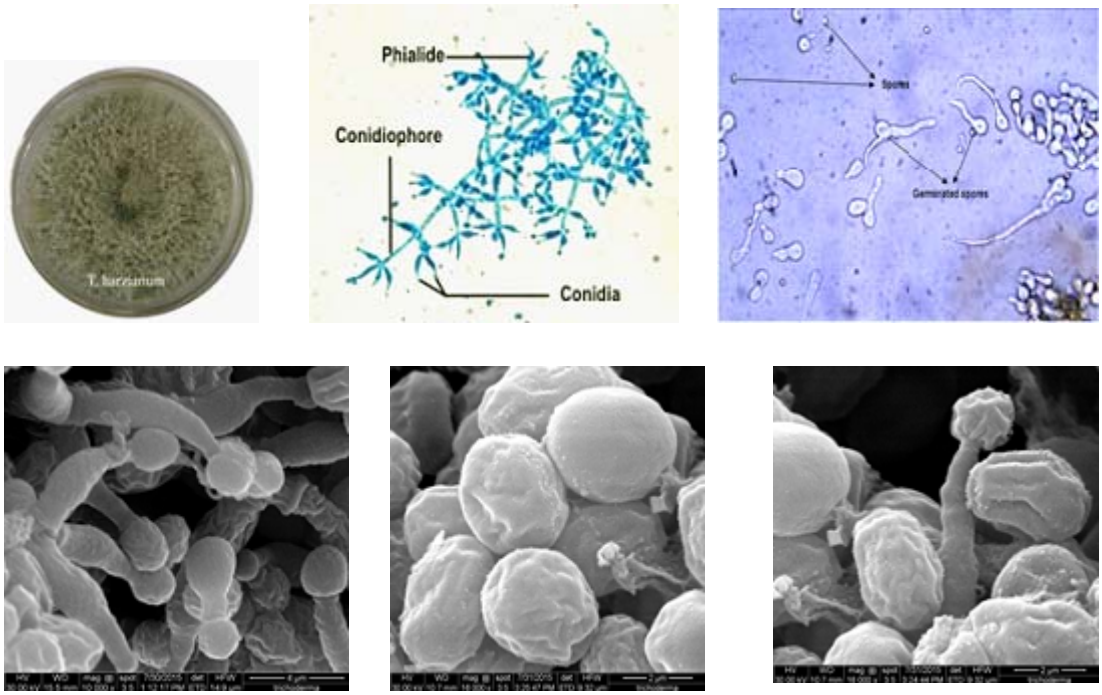

Fig. 1. Light micrographs of *T. harzianum* (*Th* Azad/6796) (A) Growth on PDA Media (B) Microscopic observation at 40x(C) Spore germination, Scanning electron micrographs of *T. harzianum* phialides at 2500x (D) group of spores at 16000x (E) full view of *T. harzianum* spores at 16000x(F)

been seen found that when *Trichoderma* is applied it enhances the host immune response. *Trichoderma* metabolites that may act as elicitors of plant resistance, or the expression in transgenic plants of genes whose products act elicitors, also results in the synthesis of phytoalexins, PR proteins and other compounds, and in an increase in resistance against several plant pathogens, including fungi and bacteria (Dana MM *et al* 2001, Elad Y, at al 2000)as well as resistance to hostile abiotic conditions.

The primers for the identified genes designed using *in silico* approach for primer designing and these are used for identifying the *Trichoderma* strains in wet lab.

The ech42 primer was used to detect the endochitinase gene in *T. harzianum Th* Azad From the figure 2, it is quite clear that the endochitinase gene is present in both species thus this can be used for the gene identification purpose.

Table 1. Genes involved in cell wall degradation in *T.harzianum*

Gene Name	Accession No.	Primer Pair (52 -32 sequences)	Melting Temperature T_m (°C)
ech-42	X79381.1	Forward - cacaagctcttgcacgatgg	56.9
		Reverse – catgctttgccaacatggtc	55.5
chit-HAR3	AB041753.1	Forward - acgtctacaacagcctggaacg	59.4
		Reverse - agcataaccttcaagttgcggt	57.9
Xyn2	EU821597.1	Forward - gtaggttacgtctgacgg	59.5
		Reverse - ccgtgaggaagcccagtc	64.1

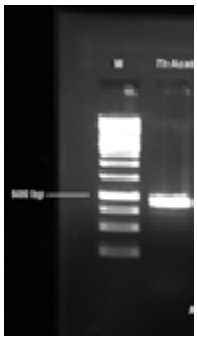

Fig 2. Amplified gene fragment of ech-42 gene present in *T. harzianum Th* Azad

The chit-HAR3 primer was used to detect the chitinase gene in *T. harzianum Th* Azad. From the figure given above, it is quite clear that the chitinase gene chit-HAR3 is present only in *T. harzianum (Th. azad)* thus this can be used for the gene identification purpose. Barley expressing

Trichoderma atroviride endochitinase *Ech42* showed increased resistance towards *Fusarium* infection (McIntyre M, *et al* 2004). Expression of the chitinase *Chit42* from *T. harzianum* in tobacco and potato plants resulted in transgenic lines highly tolerant or completely

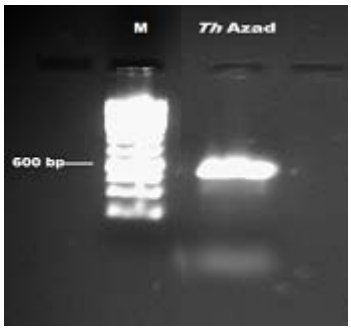

Fig. 3. Amplified gene fragment of chit-HAR3 gene present in *T. harzianum Th* Azad only

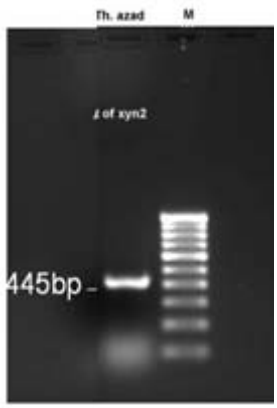

Fig. 4. *T. harzianum* (*Th.* Azad), amplified fragments with xyn2 primer

resistant to the foliar pathogens *Alternaria alternata*, *Alternaria solani* and *Botrytis cinerea* and to the soil borne pathogen *Rhizoctonia solani* (Howell CR 2003).Similar results have been obtained with the heterologous expression of *Chit42* in strawberry infected with *Colletotrichum* and with*Chit42* and a â-1, 6 glucanase in melon and tomato plants. Thus, plant protection seems to result exclusively from an increase in enzymatic activities.

The primer xyn2 was used to detect the xylanase gene *T. harzianum Th* Azad. From the figure 4, it is quite clear that the xylanase gene is present in both species thus this can be used for the gene identification purpose.

Activation of defense responses using elicitors could be a valuable strategy as an alternative to the use of transgenic plants, to protect plants against pathogens. Some authors have described that, when such plants have been in the presence of *Trichoderma*, their resistance persisted for long periods, at least several months (Harman GE, *et al* 2004) Shahid *et al*, 2014 ab).

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