Journal of
Plant Pathology & Microbiology
ISSN: 2157-7471

Shahid et al., J Plant Pathol Microb 2013, 4:10
DOI: 10.4172/2157-7471.1000204

Research Article Open Access

Morphological, Molecular Identification and SSR Marker Analysis of a Potential Strain of *Trichoderma/Hypocrea* for Production of a Bioformulation

Mohammad Shahid*, Mukesh Srivastava, Antima Sharma, Vipul Kumar, Sonika Pandey and Anuradha Singh
Biocontrol Laboratory, Department of Plant Pathology, Chandra Shekhar Azad University of Agriculture and Technology, Kanpur, 208002, India

Abstract

Seven different strains of *Trichoderma* are isolated from wilt infected leguminous crops of an Indian state and tested for their antagonistic activity against *Fusarium* (soil borne pathogen) which is expressed as a zone of inhibition in the culture plates. The seven strains are identified as *Trichoderma viride, T. harzianum, T. asperellum, T. koningii, T. atroviride, T. longibrachiatum,* and *T. virens*. Upon successful identification, morphological description and sequencing of the isolated strains with the help of universal ITS primers, the sequences are submitted to NCBI and allotted with the accession numbers JX119211, KC800922, KC800921, KC800924, KC008065, JX978542 and KC800923, respectively. Genetic variability studies reveal that a percentage of polymorphism in SSRs is obtained within the seven strains of *Trichoderma* species which is comparatively higher (>77%) than with RAPD primers (~50%). This study aims at selecting the best strain of *Trichoderma* species (*Trichoderma viride 01PP*) and then preparing a simple bioformulation that is cheap, easy to apply and readily accessible to the farmers. Shelf life of the prepared bioformulation is even checked for 180 days and it is concluded that the number of propagules start declining from 30th day onwards when the bioformulation is prepared in talc as a carrier material.

Keywords: Antagonism; Biocontrol agent; *Trichoderma*; Shelf life; Polymorphism; Genetic variability

Abbreviations: PDA: Potato Dextrose Agar; PDB: Potato Dextrose Broth; EDTA: Ethylene Diamine Tetra-Acetic Acid; w/v: weight/volume; CTAB: Cetyl Trimethyl Ammonium Bromide; PCR: Polymerase Chain Reaction; SSR: Simple Sequence Repeats; TAE: Tris base, Acetic acid and EDTA; CMC: Carboxy Methyl Cellulose; CSAU: Chandra Shekhar Azad Agriculture University; BCA: Biological Control Agent; RFLP: Restriction Fragment Length Polymorphism; ITCC: Indian Type Culture Collection; NCBI: National Centre for Biotechnology Information; ITS: Internal Transcribed Spacer; LDPE: Low Density Poly Ethylene

Introduction

The genus *Trichoderma* has its own significance in the agricultural industry due to its varied activities ranging from being a valuable antagonist against the soil-borne pathogens to acting as a provider of nutrition to the soil as well. Several scientists have worked on how this genus acts as a potential biocontrol agent against a range of pathogenic fungi. Harman et al. [1] have even reported *Trichoderma* as opportunistic, avirulent plant symbionts. They have explained the features of *Trichoderma* as to how it colonizes the roots that eventually proves beneficial to the soil in terms of nutrition and plant growth increasing crop productivity simultaneously.

The biocontrol activity of *Trichoderma* is of immense importance not only to agriculture and its crops but also the environment as it does not accumulate in the food chain and thus does no harm to the plants, animals and humans [2]. The genes and gene products involved in the biocontrol mechanism of *Trichoderma* provide a vast array of research to the scientists in Biotechnology and Bioinformatics as well.

The infrageneric classification by Bisset [3] shows significant morphological similarities between *Trichoderma* and *Hypocrea* and have defined genus *Trichoderma* to include the anamorphs of *Hypocrea*.

The morphology of *Trichoderma* spp. is very interesting to study as there are a finite number of morphological descriptors to study and disseminate the genus and its features [4,5]. It is believed that the identification of any microorganism becomes quite easy by a careful morphological observation; hence, a detailed morphological description of some of the commercially important strains of *Trichoderma* has been carried out in this study. Samuels [6] described the systematics, the sexual stage and the ecology of *Trichoderma* and mentioned in his study that the morphology of *Trichoderma* is not only limited to a few characters but many species may be included in this genus due to their geographical distribution.

Druzhinina and Kubicek [7] studied and brought forth the species concepts and biodiversity in *Trichoderma* and *Hypocrea* by aggregating the morphological, physiological and genetic studies and presented an update on the taxonomy and phylogeny of a number of taxa. This helped us in understanding that the identification of *Trichoderma* only on the basis of morphology is not of high precision. Thus, molecular identification and characterization comes under investigation that would help in evaluating the genetic diversity between the species.

Kumar et al. [8], Shahid [9] and Sagar et al. [10] focused on the molecular identification and analysis of the genetic variability of a specific strain of *Trichoderma* based on antagonistic and RAPD analysis in some leguminous crops (Pigeonpea, Chickpea and Lentil) produced in Uttar Pradesh (India). RAPD analysis with a set of 20 OPA primers was carried out on 5 isolates of the same strain (*Trichoderma longibrachiatum*) collected from different soil samples of Pigeonpea. This resulted in a significant amount of genetic variability where more

***Corresponding author:** Mohammad Shahid, Biocontrol Laboratory, Department of Plant Pathology, CSA University of Agriculture and Technology, Kanpur, India, E-mail: shahid.biotech@rediffmail.com

Received : September 10, 2013; **Accepted** October 06, 2013; **Published** October 10, 2013

Citation: Shahid M, Srivastava M, Sharma A, Kumar V, Pandey S, et al. (2013) Morphological, Molecular Identification and SSR Marker Analysis of a Potential Strain of *Trichoderma/Hypocrea* for Production of a Bioformulation. J Plant Pathol Microb 4: 204. doi:10.4172/2157-7471.1000204

Copyright: © 2013 Shahid M, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

than 50% of the amplified fragments in each case were polymorphic. Thus, it was concluded that there was good genetic variability among the isolates under study.

Based on the genetic variability studies done earlier, the study now focuses upon developing a strain-specific molecular marker solely for the identification of *Trichoderma* species. rRNA based analysis is thought to be the best one to explore the microbial diversity and identify new strains [11].

The study also includes the behavior of these BCAs against fungal wilt pathogens affecting leguminous crops. *Fusarium* wilt causes huge loss to the leguminous crops in India every year ranging from 15 to 20% thereby reducing the production of important legumes. Various management strategies such as use of resistant cultivars are been undertaken to prevent the crops and soils as well from the wilt caused by *Fusarium* as it may last for several years. Thus, it becomes necessary to derive a cheap and better way to fight against the pathogen and increase the crop production. Bioformulation containing *Trichoderma* has emerged as an effective alternative to this problem and thus has been disseminated in this report. But, before preparing a bioformulation with *Trichoderma*, the effect of media, temperature and pH on the growth and sporulation of *Trichoderma* species should be known [12,13]. *Trichoderma species*, when grown either in PDA or PDB within a pH range of 7-7.5 and at an optimum temperature range of 25-30˚C gives the best growth and sporulation rates both.

Talc-based bioformulation of *Trichoderma* [14] has proven beneficial to the wilt infected leguminous crops but an important aspect to be taken into prior consideration is the shelf life of spores that are present in talc. Various methods and measures are still to be taken that can result in the longevity, competitiveness and survival of *Trichoderma* on fields.

Materials and Methodology

Isolation and selection of strains

Trichoderma strains were isolated from the soil of pulse fields of various districts of Uttar Pradesh (India) and were tested against phythopathogenesis. The most promising isolates were selected for biochemical, molecular and disease suppressiveness tests. Initially, a total of seven strains were identified and were selected for further study. Based on the descriptions of Bissett [3], we classified these fungi as: *Trichoderma* anamorph and *Hypocrea* teleomorph. The isolates were screened for antagonistic activity towards the major soil borne fungi such as *Fusarium solani, Rhizoctonia solani, Pythium ultimum, Macrophomina phaseolina, Sclerotinia sclerotiorum, Phytophthora*, *Fusarium oxysporum* and *Sclerotium cepivorum* that were previously isolated and identified in the Biocontrol Laboratory, Department of Plant Pathology, CSAUAT, Kanpur, India.

in vitro bioassay

in vitro bioassay was conducted between the *Trichoderma* isolates and the phytopathogenic fungi in petridishes containing PDA. Isolates which showed a marked effect towards pathogens were selected and used for further study. Each *Trichoderma* isolate was separately inoculated into 100 ml Potato Dextrose Broth and incubated at 20˚C for 10 days. After incubation, the cultures were filtered through 0.22 mm Millipore filters and the aliquots (2 ml) of these filtrates were placed in sterile petridishes and 25 ml of 1/4 strength PDA at 45˚C was added. Once the agar solidified, mycelial discs of the pathogens (7 mm in diameter) obtained from actively growing colonies were placed gently on the centre of the agar plates. The petridishes were incubated at 20°C for 6 days. There were three replicates for each experiment and the growth reduction of the pathogens was recorded.

Morphological descriptors such as colony morphology, colony color, colony edge and others of each strain were studied.

SSR analysis

DNA was extracted using CTAB method from all seven isolates and quantified using agarose gel electrophoresis. A total of 20 SSR primers i.e., SSR 1-20 were selected. PCR was programmed with an initial denaturing for 4 minutes at 94°C; followed by 35 cycles of denaturation for 1 minute at 94°C; annealing at 36°C for 1 minute; extension for 90 seconds at 70°C, and a final extension for 7 minutes at 72°C in a Primus 96 advanced gradient Thermocycler. PCR product (20 µl) was mixed with loading buffer (8 µ1) containing 0.25% Bromophenol Blue, 40% (w/v) sucrose in water and then loaded in 2% agarose gel with 0.1% ethidium bromide for examination by horizontal electrophoresis.

Electrophoresis

The amplification products were analyzed by electrophoresis according to Sambrook and Russell [15] in 2% agarose in TAE buffer (for a litre of 50X TAE Stock solution, we used: 242 g Tris Base, 57.1 ml Glacial Acetic Acid and 100 ml 0.5 M EDTA), stained with 0.2 µg/ml ethidium bromide. Nucleic acid bands were photographed and detected by BioRad Gel Doc system.

Preparation of bioformulation

Talc powder was evaluated as carrier material to produce bioformulation of *Trichoderma sp*. The carrier was dried under sun, powdered (sieve pore, 1mm) and sterilized at 1.05 kg/cm^2 pressure for 30 min. The substrate was mixed with 7 days old culture of respective *Trichoderma* spp. which were previously grown on potato dextrose agar in 2:1 (solid culture) w/v and CMC 5 gm/kg was added as adjuvant. Fifty grams of such mixture was then filled in polypropylene bags (25x30 cm) tied and stored at 25 ± 2˚C. Observations on colony forming units (cfu) of *Trichoderma spp.* was recorded initially and at monthly interval up to 6 months for shelf life study.

Seed treatment

Required quantity of fungicide (*Vitavax* @ 2 gm/kg seed), insecticide (Chloropyriphos 20 EC @ 8ml/kg seed), biocontrol agent (*Trichoderma viride* @ 4 gm/kg seed) and biofertilizer *Rhizobium* culture @ 1 packet/acre or 30 gm/kg seeds) along with different combination with 100 seeds of lentil and Chickpea taken from the healthy fields and 100 seeds of lentil and chickpea taken from the infected fields were used for studies.

Results

Isolation and bioassay

A total of seven isolates of *Trichoderma species* were isolated from the soil of pulse fields of various districts of Uttar Pradesh, India. These include *Trichoderma viride*, *T. harzianum, T. asperellum, T. koningii, T. atroviride*, *T. longibrachiatum* and *T. virens*.

All tested strains in genus *Trichoderma* had high or moderate antagonistic activity towards pathogens expressed as a zone of inhibition and fungal growth reduction by using culture filtrate. Among all isolated strains, *T. harzianum* and *T. viride* were found to be the most effective species against all pathogens.

Trichoderma strains that were isolated and taken into consideration in this study have been validated and submitted to the Indian Type

Strain No.	Name of Bioagent	ITCC Accession No	GenBank Accession No.	Strain code	Source
T1	*T. viride*	8315	JX119211	*01PP*	Hardoi (U.P., India)
T2	*T. harzianum*	6796	KC800922	*Th azad*	CSA, Kanpur (U.P., India)
T3	*T. asperellum*	8940	KC800921	T_{asp}/CSAU	CSA, Kanpur (U.P., India)
T4	*T. koningii*	5201	KC800924	T_k (CSAU)	CSA, Kanpur (U.P., India)
T5	*T. atroviride*	7445	KC 008065	*71L*	Hardoi (U.P., India)
T6	*T. longibrachiatum*	7437	JX978542	*21PP*	Kaushambi (U.P., India)
T7	*T. virens*	4177	KC800923	T_{vi} (CSAU)	CSA, Kanpur (U.P., India)

Table 1: Details of *Trichoderma* strains.

Name of Strains	Colony Growth rate (cm/day)	Colony color	Reverse color	Colony edge	Mycelial form	Mycelial color	Conidiation	Conidiophore branching	Conidia wall	Conidial color	Chlamydospores
T. viride	8-9 in 3 days	Dirty green	Dark greenish	Smooth	Floccose to Arachnoid	Watery white	Ring like zones	Ball like structure	Rough	Green	Not observed
T. harzianum	8-9 in 3 days	Dark green	Colorless	Wavy	Floccose to Arachnoid	Watery white	Ring like zones	Highly branched, regular	Smooth	Dark Green	Not observed
T. asperellum	5-6 in 3 days	Snow white green	Orange	Smooth	Floccose	Watery White	Ring like zones	Branched, regular	Smooth	Green	Not observed
T. koningii	7-8 in 3 days	Dirty green	Yellowish	Smooth	Floccose to Arachnoid	Watery white	Ring like zones	Highly branched, regular	Rough	Grayish Green	Not observed
T. atroviride	5-6.5 in 3 days	Light dark effuse	Colorless	Effuse	Floccose to Arachnoid	Watery white	Irregular	Irregular	Rough	Yellowish Green	Not observed
T. longibrachiatum	8-9 in 4 days	White to green	Colorless	Effuse	Floccose to Arachnoid	Watery white	Circular zones	Rarely re-branched	Smooth	Green	Not observed
T. virens	8-9 in 3 days	Snow white	Colorless	Smooth	Floccose to Arachnoid	Watery White	Flat	Highly branched, regular	Smooth	Dirty Green	Not observed

Table 2: Morphological descriptors used for the characterization of native isolates of *Trichoderma* spp.

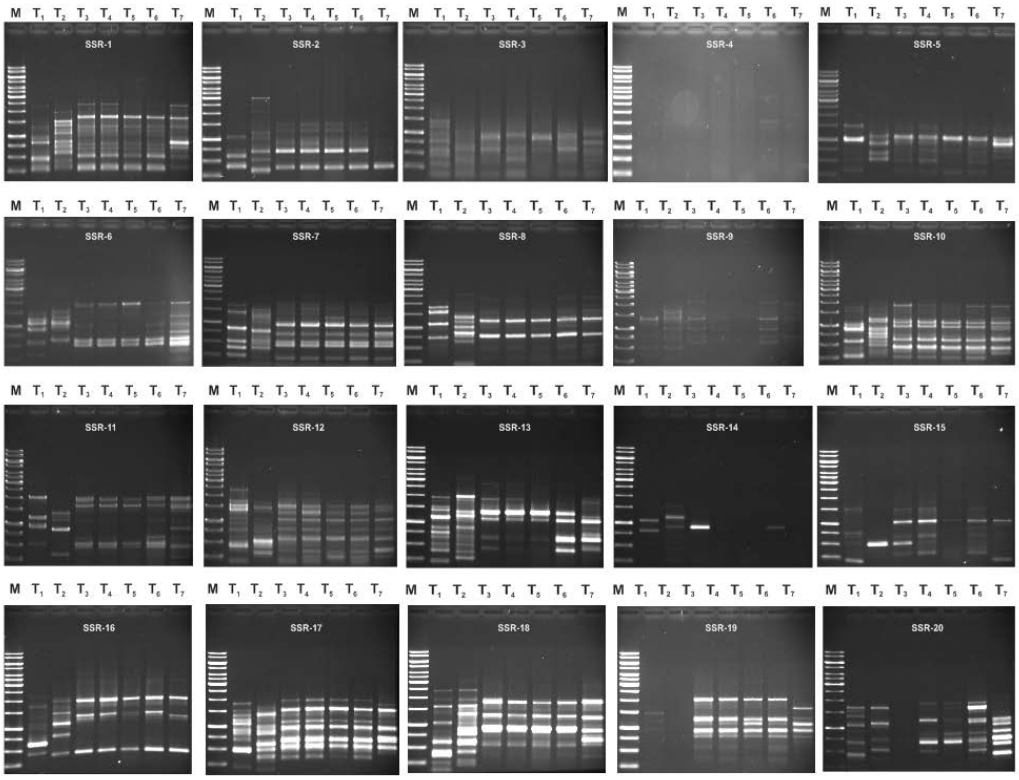

Figure 1: Molecular Analysis of Trichoderma spp. using SSR Primers.

Name of SSR Primer	Primer Sequence (5' – 3')	Amplified product	Total no. of bands	No. of Polymorphic bands	No. of Monomorphic bands
SSR 1.	F: GAAACAACACCGAAATACAC R: CAAGTCAGATGAAGTTTG	YES	6	2	4
SSR 2.	F: GACTCATACTTTGTTCTTAGCAG R: GAACGGAGCGGTCACATTAG	NO	-	-	-
SSR 3.	F: CAAGCTGACGCCTATGAAGA R: CTTTCACTCACTCAACTCTC	YES	4	3	1
SSR 4.	F: CATGGTGGAATAGTGATGGC R: CTCCATACACCACTCATTCAC	YES	4	3	1
SSR 5.	F: CCAAATACTGCAACACACCG R: GTTCCCATCAAGGCAGAAGG	NO	-	-	-
SSR 6.	F: CCATGCATACGTGACTGC R: GTTGACTGTTGGTGTAAGTG	YES	6	3	3
SSR 7.	F: GTTATCTTCCAGCGTC R: GATATACAATCAGAGATG	NO	-	-	-
SSR 8.	F: GGGAATTTGTGGAGGGAAG R: CCTCAGAATGTCCCTGTC	YES	3	1	2
SSR 9.	F: GCGGCGAGCAAATAAAT R: GGAGAATAAGAGTGAAATG	NO	-	-	-
SSR 10.	F: CCGTAAGAATAGGTGTC R: GGAAAATAGGGTGGAAAG	YES	7	2	5
SSR 11.	F: GAACTCAGTTTCTCATTG R: GAACATATCCAATTATCATC	YES	10	10	0
SSR 12.	F: GTATGTGCTTGTATGCTTC R: GAACGGAGCGGTCACATTA	NO	-	-	-
SSR 13.	F: CCACGTATGTGACTGTATG R: GAAAGAGAGGCTGAAACTTG	YES	12	11	1
SSR 14.	F: GGTAGGTGAGATAGTTG R: GGAGCAAGAAGAAGCAG	YES	11	11	0
SSR 15.	F: GGAATTTATCACACTATCTC R: GACTCCCAACTTGTATG	YES	7	7	0
SSR 16.	F: GTACATTGAACAGCATCATC R: CAATAGGGCATGAAAGGAG	NO	-	-	-
SSR 17.	F: CACATATGAAGATTGGTCAC R: CATTTATGTCTCACACACAC	NO	-	-	-
SSR 18.	F: GTGTGTACCTAAAGCCTTG R: GTAAGTTGATCAAACGCCC	YES	5	3	2
SSR 19.	F: GTGTGCATGGTGTGTG R: CCATCCCCCTCTATC	NO	-	-	-
SSR 20.	F: CACGACTATCCCACTTG R: CTTACTTTCTTAGTGCTATTAC	YES	9	9	0
GRAND TOTAL			**84**	**65**	**19**

Table 3: SSR Amplification and their corresponding PCR products for bioagent *Trichoderma spp*

Culture Collection (ITCC) and GenBank (NCBI) database where particular accession numbers have been allotted to the specific strain of each species (Table 1).

Morphological description

Morphological study of the *Trichoderma* strains has been done and the characteristics include various parameters such as colony growth rate, colony color, colony edge, mycelial form, growth pattern and speed along with morphology of conidia and phialids, conidia color, shape and size etc. were studied for the identification of each strain of the genus *Trichoderma* (Table 2).

Molecular characterization of *Trichoderma spp.* using SSR markers

A set of 20 SSR primers were used in this study (Table 3). The preliminary studies indicate that the *Trichoderma* spp. isolates under study had very good diversity and there is a strong possibility to get the isolate-specific primers that will be utilized for identification of the particular *Trichoderma* isolates with a good biological potential from the field isolates without undergoing the cumbersome bioassay. All reproducible polymorphic bands were scored and analyzed following UPGMA cluster analysis protocol and computed *in silico* into similarly matrix using NTSYSpc.

The size of the fragments (molecular weight in base pairs) was estimated by using 1 kb ladder marker which was run along with the amplified products. In the gel, "1" indicates the presence of a band whereas "0" indicates the absence of any band. Out of the seven strains of *Trichoderma* spp. tested, the percentage of polymorphism in SSRs obtained was more than 77%. This shows that there is a complete variability within the strains of *Trichoderma* spp. being isolated from different fields of the Indian State (Uttar Pradesh). This would enable us to develop a potential strain possessing competitive ability, growth promoting characters and inducing resistance in plants (Figure 1).

In the dendrogram shown in Figure 2, all the 7 isolates of *Trichoderma spp.* were distinctly divided into two major clusters **A** and B at 20 units. Isolate T_k/*CSAU* and *01PP* spanned the extremes of the entire dendrogram. Genetic dissimilarity ranged from a lowest value of 0.143 to a highest value of 0.857 (between T_{asp}/*CSAU* and T_k *(CSAU)*. Isolates T_k *(CSAU)*, *71L*, *21PP*, and T_{vi} *(CSAU)* were assigned to cluster

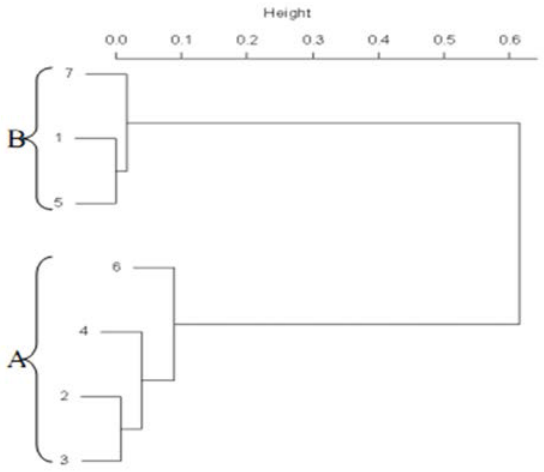

Figure 2: Dendrogram for *Trichoderma spp*. isolates as revealed by SSR markers; where: 1: *Th azad, 2: 71L, 3: 21PP, 4: T_k (CSAU), 5: T_{asp}/CSAU, 6: T_{vi} (CSAU), 7: 01PP.*

Strain Name	Locus	Definition	Primer used	Sequence bp
T. viride 01PP	JX119211	*Hypocrea rufa* isolate 01PP-8315/11 18S ribosomal RNA gene, partial sequence; Internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28 S ribosomal RNA gene, partial sequence.	ITS-1tccgtaggtgaacctgcgg ITS-2 tcctccgcttattgatatgc	1173bp
T. harzianum Th azad	KC800922	*Trichoderma harzianum* isolate *Th-azad/CSAU* 6796 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 4, partial sequence.	ITS-1agagtttgatcctggctcag ITS-4ggttaccttgttacgactt	546bp
T. asperellum T_{asp}/CSAU	KC800921	*Trichoderma asperellum* isolate T_{asp}(CSAU)-8940 18s ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8s ribosomal RNA gene, and internal transcribed spacer 4, partial sequence	ITS-1tccgtaggtgaacctgcgg ITS-4 tcctccgcttattgatatgc	641 bp
T. koningii T_k (CSAU)	KC800924	*Trichoderma koningii* isolate *T_k (CSAU)* 5201 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 4, partial sequence.	ITS-1 tctgtaggtgaacctgcgg ITS-4 ggaagtaaaagtcgtaacaagg	206 bp
T. atroviride 71L	KC008065	*Trichoderma atroviride* strain TAU8 18S ribosomal RNA, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA, and internal transcribed spacer 2 complete sequence; and 28S ribosomal RNA partial sequence.	ITS-1tcctccgcttattgatatgc ITS-2 ggaagtaaaagtcgtaacaagg	627bp
T. longibrachiatum 21PP	JX978542	*Trichoderma longibrachiatum* strain 21PP 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	ITS-1tcctccgcttattgatatgc ITS-2 ggaagtaaaagtcgtaacaagg	664 bp
T. virens T_{vi} (CSAU)	KC800923	*Trichoderma virens* isolate T_{vi} (CSAU)-417718S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 4, partial sequence.	ITS-1 tcctccgcttattgatatgc ITS-4 ggaagtaaaagtcgtaacaagg	635 bp

Table 4: Submission of the gene sequences at NCBI Database.

'**A**'. Genetic dissimilarity among the entries in this cluster ranged from a lowest of 14.3% (between *21PP* and *T_{vi} (CSAU)* to a highest of 35.7% (between *T_{vi} (CSAU)* and *T_k (CSAU)*. The other cluster 'B' comprised of three isolates namely *Th azad*, *T_{asp} (CSAU)* and *01PP* were grouped together. The genetic dissimilarity in this group ranged from 33.3% between *T_{asp} (CSAU)* and *01PP* to 75% between *Th azad* and *01PP*.

The molecular identification and characterization of *Trichoderma* isolates was conducted with the help of universal ITS primers also. Four ITS primer sequences were used for the identification of all seven isolates of *Trichoderma* and were then submitted to NCBI database. The details of the strains submitted can be seen in Table 4.

Bioformulation and its validation under *in vitro* conditions

Talc-based bioformulation of *Trichoderma* is prepared as it is relatively cheap and easily accessible to farmers for use on fields. It can be stored in plastic bags for long as it has been observed that storing the talc-based bioformulation in plastic bags increases the shelf-life of *Trichoderma* preserving its bioefficiency simultaneously.

The shelf life of all the seven isolates was also ascertained at ambient environment prevailing during a period of 6 months on the basis of spore load per gram. The talc based powder of the bioagent was prepared (*Trichoderma viride 01PP* (spore+mycelium) 1.0% w/w+Talc 98.5% w/w+0.5% carboxyl methyl cellulose) and used for shelf life, bioefficacy etc. studies. The talc based bioformulation was stored in LDPE pouches. The powder was dull white in color, pH 7.0, moisture 8% and cfu of 29.7×10^6. It was found that the bioformulation has good shelf life up to six months and then the spores started declining.

Shelf life of *Trichoderma* in talc as a carrier material was determined

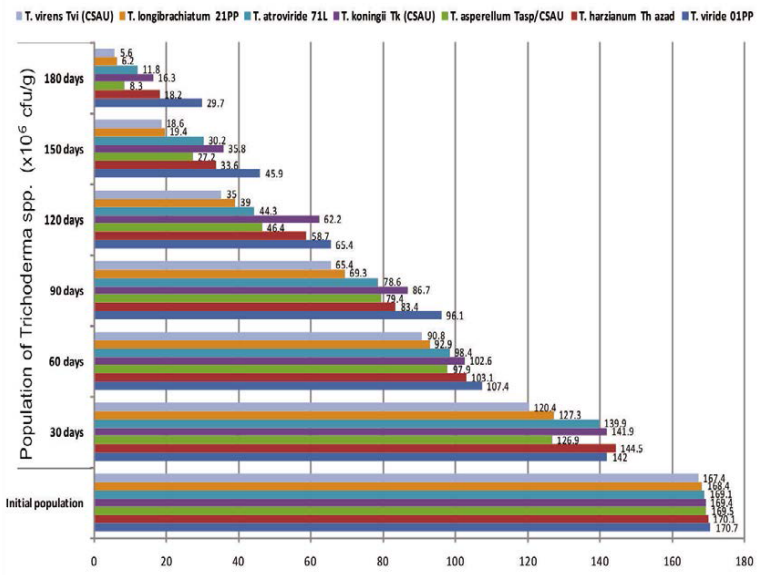

Figure 3: Effect of Talc as a carrier on the population of *Trichoderma spp.*

Sl. No.	Treatment	Germination %	Root length (cm)	Shoot length (cm)	Seedling length (cm)	Dry weight	Vigour index-I	Vigour index-II
1.	*Trichoderma viride*	86.67	7.58	11.70	19.28	0.14	1671.00	12.13
2.	*Vitavax*	85.00	7.31	11.26	18.57	0.13	1578.45	11.05
3.	*Chlorpyriphos*	81.00	7.27	8.87	16.14	0.11	1307.34	8.91
4.	*Rhizobium*	81.33	6.45	9.69	16.14	0.11	1312.67	8.95
5.	*Trichoderma viride+Vitavax*	90.00	7.91	13.08	20.99	0.14	1889.10	12.60
6.	*Trichoderma viride+Chlorpyriphos*	77.67	5.90	10.41	16.31	0.11	1266.80	8.54
7.	*Trichoderma viride+Rhizobium*	84.33	6.59	9.35	15.94	0.11	1344.22	9.28
8.	*Vitavax+Chlorpyriphos*	67.67	5.51	9.35	14.86	0.12	1005.58	8.12
9.	*Vitavax+Rhizobium*	73.67	4.41	8.34	12.75	0.11	939.29	8.10
10.	*Chlorpyriphos+Rhizobium*	84.67	3.95	7.16	11.11	0.11	940.68	9.31
11.	*Trichoderma viride+ Vitavax+Chlorpyriphos*	77.33	6.57	10.85	17.42	0.12	1347.09	9.28
12.	*Trichoderma viride+ Vitavax+Rhizobium*	78.33	5.84	8.92	14.76	0.12	1156.15	9.40
13.	*Trichoderma viride+ Chlorpyriphos+ Rhizobium*	80.00	5.57	9.74	15.31	0.11	1224.80	8.80
14.	*Vitavax+Chlorpyriphos+Rhizobium*	79.33	5.70	8.83	14.53	0.10	1152.66	7.93
15.	*Vitavax+Chlorpyriphos+Vitavax+Rhizobium*	74.00	5.45	9.05	14.5	0.10	1073.00	7.40
16.	Control	66.33	3.87	7.10	10.97	0.10	727.64	6.63
	CD=5%	5.69	1.22	0.63	1.72	0.02	321.60	1.81
	S.D.	2.79	0.60	0.31	0.86	0.01	157.88	2.03

Table 5: Evaluation with special reference to the use of pesticides in seed treatment in combination with bioagents.

at a time interval of 30 days that further indicated that the number of propagules started declining from 30th day onwards. Talc-based bioformulation was found to be the best material to retain maximum number of viable propagules i.e., 29.7×10^6 cfu/g at 180 days of storage. It has also been found that the isolates can retain their viability up to 120 days in all the cases (Figure 3).

Under natural conditions, application of talc-based solid formulation of *Trichoderma* in soil provides protection against wilt disease in leguminous crops. Higher reduction in wilt was obtained in lentil and pigeon pea crops. As compared with the control and other strains, application of *Trichoderma viride 01PP* was more effective in reducing the wilt disease caused by *Fusarium* in Pigeonpea. *Trichoderma* species can act as biocontrol agents through different synergistic mechanisms. However, it is difficult to predict the degree of synergism and the behavior of a BCA in natural system. Considering that the environmental conditions are important, the right selection of BCAs, which begins with a safe characterization of biocontrol strains in the new taxonomic schemes of *Trichoderma*, is equally important since the exact identification of strains at the species level is the first step in utilizing the full potential of fungi in specific applications. *Trichoderma* species play an important role in controlling fungal plant pathogens, especially soil borne fungal pathogens. Strains of *Trichoderma* can produce extracellular enzymes and antifungal antibiotics, they may also be competitors to fungal pathogens, promote plant growth, and induce resistance in plants.

The different pre-sowing seed treatments when taken from healthy fields showed different response for all seven seed quality attributes i.e. germination, root length, shoot length, seedling length, dry weight, vigour index I and vigour index II. The data revealed that *Vitavax* followed by treatment with *Trichoderma viride* were found superior

(Table 5). Among all the treatments, control exhibited the poorest performance in most of the characters under study.

The commercial use of *Trichoderma* BCAs must be preceded by precise identification, adequate formulation, and studies about the synergistic effects of their mechanisms of biocontrol. *T. viride 01PP* have been reported as the most important BCAs against plant pathogenic fungi. The strain distribution in several genotypes could also support the idea of developing antifungal formulations in which different *Trichoderma* BCAs could be combined. The use of *Trichoderma*-based products is not only safe for the farmers and consumers but it also proves friendly to the environment.

Discussion

The morphological characters of the fungus under study agree very closely with the description given by Vasudeva and Srinivasan [16] and Booth [17]. Cornea et al. [18] found that the molecular analysis using ITS-RFLP and PCR with specific primers allow the confirmation of previous taxonomic determination of *Trichoderma harzianum* and *T. viride*. However, an increased intra-specific molecular polymorphism was observed using several arbitrary primers (RAPD) analysis. Shahid et al. [19] and Singh et al. [20] also reported that germination and seedling length along with seedling dry weight are important attributes, which determine the quality of seed of any seed lot. Besides these quality seed parameters seed vigour index also plays very crucial role in predicting the fate of any seed lot under biotic and abiotic stress conditions.

Conclusion

It is concluded from this study that *Trichoderma* has been successfully isolated, identified, characterized and used as an effective biocontrol agent against wilt caused by other pathogenic fungi. The seven strains of *Trichoderma* have been isolated from wilt infected leguminous crops and tested in the laboratory for the identification of pathogens infecting the crops.

The strains have been examined morphologically and at molecular level as well. Specific markers have been defined that could quickly identify specific strains and amplify them. The genetic variability among the strains is also studied with the help of a set of SSR markers. The effect of enzyme activities during interaction with the pathogen is also counted and the data reveals the best carbon source for the enzyme for its induction.

In the end, a talc based bioformulation is prepared that showed beneficial effects when applied on wilt infected crops on pulse fields.

Acknowledgement

The authors are grateful to the financial support granted by the Indian Council of Agricultural Research under the Niche Area of Excellence on “Exploration and Exploitation of Trichoderma as an antagonist against soil borne pathogen” running in Biocontrol Laboratory, Department of Plant Pathology, Chandra Shekhar Azad University of Agriculture and Technology, Kanpur-208002, Uttar Pradesh, India.

References

1. Harman GE, Howell CR, Viterbo A, Chet I, Lorito M (2004) *Trichoderma* species—opportunistic, avirulent plant symbionts. Nat Rev Microbiol 2: 43-56.
2. Monte E, Llobell A (2003) *Trichoderma* in organic agriculture. V Congreso Mundial del Aguacate 725-733.
3. Bissett J (1991) A revision of the genus *Trichoderma* . II. Infrageneric classification. Canadian Journal of Botany 69: 2357-2372.
4. Gams W, Bissett J (1998) Morphology and identification of *Trichoderma*. In: Kubicek C and Harman G (Eds). *Trichoderma* and *Gliocladium*. Taylor & Francis, London, UK.
5. Gams W, Meyer W (1998) What exactly is *Trichoderma harzianum*? Mycologia 90: 904-915.
6. Samuels GJ (2006) *Trichoderma*: systematics, the sexual state, and ecology. Phytopathology 96: 195-206.
7. Druzhinina I, Kubicek CP (2005) Species concepts and biodiversity in *Trichoderma and Hypocrea*: from aggregate species to species clusters? J Zhejiang Univ Sci B 6: 100-112.
8. Kumar V, Shahid M, Singh A, Srivastava M, Biswas SK (2011) RAPD Analysis of *Trichoderma longibrachiatum* isolated from Pigeonpea Fields of Uttar Pradesh. Indian J Agric Biochem 24: 80-82.
9. Shahid M (2012) Evaluation of Antagonistic activity and Shelf life study of *Trichoderma viride* (01 PP-8315/11). Advances in Life Sciences 1: 138-140.
10. Sagar MSI, Meah MB, Rahman MM, Ghose AK (2011) Determination of genetic variations among different Trichoderma isolates using RAPD marker in Bangladesh. J Bangladesh Agril Univ 9: 9-20.
11. Shahid M (2013) Sequencing of 28SrNA Gene for Identification of *Trichoderma longibrachiatum* 28 CP/ 74444 Species in Soil Sample. International Journal of Biotechnology for Wellness Industries 2: 84-90.
12. Singh A, Shahid M, Pandey NK, Kumar S, Srivastava M, Biswas SK (2011) Influence of temperature, pH and media for growth and sporulation of *Trichoderma atroviride* and its Shelf life study in different carrier based formulation. J Pl Dis Sci 6: 32-34.
13. Shahid M, Singh A, Srivastava M, Sachan CP, Biswas SK (2011) Effect of seed treatment on Germination and Vigour in Chickpea. Trend in Biosciences 4: 205-207.
14. Shahid M (2012) Molecular characterization and variability of *Trichoderma longibrachiatum* based on antagonistic and RAPD analysis in legume crops of Uttar Pradesh. J Botan Soc Bengal 66: 105-110.
15. Sambrook J, Russell DW (2001) Agarose Gel Electrophoresis. CSH Protocols.
16. Vasudeva RS, Srinivasan KV (1952) Studies on the wilt disease of lentil *(Lens esculenta Moench)*, Indian Phytopath 5: 23-32.
17. Booth C (1971) The genus *Fusarium*. CMI, Kew, Surrey, England.
18. Cornea CP, Pop A, Matei S, Ciuca M, Voaides C, et al. (2009) Antifungal action of new *Trichoderma* species Romanian isolates on different plant pathogens. Biotechnol and Biotechnol 23: 766-770.
19. Shahid M, Singh A, Srivastava M, Mishra RP, Biswas SK (2011) Effect of temperature, pH and media for growth and sporulation of *Trichoderma longibrachiatum* and self life study in carrier based formulations. Ann Pl Protec Sci 19: 147-149.
20. Singh A, Shahid M, Sachan CP, Srivastava M, Biswas SK (2013) Effect of seed treatment on Germination and Vigour in Lentil. J Pl Dis Sci 8: 124-125.