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Full Length Research Paper

Production of a novel bioformulation of *Trichoderma/Hypocrea* using biotechnological approaches

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Seven different strains of *Trichoderma* isolated from wilt infected legume fields of UP State were tested for their antagonistic activity against *Fusarium* (soil borne pathogen), which is expressed as a zone of inhibition in the culture plates. Seven strains were identified as *Trichoderma viride, Trichoderma harzianum, Trichoderma asperellum, Trichoderma koningii, Trichoderma atroviride, Trichoderma longibrachiatum* and *Trichoderma virens*. Upon successful identification, morphological description and sequencing of the isolated strains with the help of universal Internal transcribed spacer (ITS) primers, the sequences are submitted to NCBI and allotted with the accession numbers JX119211, KC800922, KC800921, KC800924, KC008065, JX978542 and KC800923, respectively. Mycoparasitism is an inherent ability of the genus *Trichoderma* that involves the role of cell wall degrading enzymes such as xylanase, glucanase, chitinase, and proteinase etc. and acting as a biocontrol agent. This study reports the xylanase enzyme activity for all the isolated strains on a media containing birchwood xylan as a carbon source where *T. harzianum (Th* Azad), could show maximum activity there upon acting as a strain of utmost importance to the farmers for protecting their crops against wilting. Genetic variability within different strains of the same species was also analyzed to develop a novel strain possessing competitive ability, growth promoting characters and inducing resistance in plants. A percentage of polymorphism in Simple sequence repeats (SSRs) is obtained within the seven strains of *Trichoderma* species, which is comparatively higher (>77%) than with Random amplified polymorphic DNA (RAPD) primers (~50%). The study aims at exploring *Trichoderma* species and then preparing a simple bioformulation that is cheap, easy to apply and readily accessible to the farmers. The shelf life of *Trichoderma* in 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. It was also found that seed treatment with the bioformulation *T. harzianum* (*Th* Azad) (5 g/kg seeds) could increase seed germination, root and shoot length and seedling vigour over untreated ones.

Key words: Antagonism, bio-control agent, *Trichoderma*, shelf life, polymorphism, genetic variability.

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. (2004) 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 (Monte and Llobell, 2003). 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.

Various strains of *Trichoderma* have been isolated and identified till date in an attempt to investigate the morphology, molecular characterization, phylogeny, taxonomy, mode of action, enzymatic activity, antagonistic activity, bioformulation, etc. which has opened the gates of new research on this topic. Most ongoing research is aimed at developing new techniques or strains that can prove ecologically beneficial to the agriculture.

The intrageneric classification by Bisset (1991) shows significant morphological similarities between *Trichoderma* and *Hypocrea* and have defined genus *Trichoderma* to include the anamorphs of *Hypocrea*.

The morphology of *Trichoderma* sp. is very interesting to study as there are a finite number of morphological descriptors to study and disseminate the genus and its features (Gams and Bissett, 1998; Gams and Meyer, 1998). 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 (2006) 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.

Isolation, characterization and morphological description of *Trichoderma* species are important before further dissemination is done leading to the biomass production at different environmental and cultural conditions. An attempt has been made to grow different species of *Trichoderma* at varying pH, temperature and agitation speeds in order to reveal all the relevant and favorable parameters.

The isolates from the soils of legume fields are more adaptive to the tested pH ranges than the isolates from virgin soils where there is no intervention of agricultural practices. As *Trichoderma* is an ecofriendly biological control agent (BCA) against other soil borne plant pathogens, it is necessary to grow it at suitable conditions before it is used for commercial purposes. Different pH, temperatures and agitation speeds have been tested in this study for a better growth of different isolates of *Trichoderma* species.

Druzhinina and Kubicek (2005) 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. (2011), Shahid et al. (2012a) and Sagar et al. (2011) focused on the molecular identification and analysis of the genetic variability of a specific strain of *Trichoderma* based on antagonistic and Random amplified polymorphic DNA (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 species (*Trichoderma longibrachiatum*) collected from different soil samples of pigeon pea. This resulted in a significant amount of genetic variability where more 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 was focused 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 (Shahid et al., 2013).

The genus *Trichoderma* is well known among the microbial world as BCA and this is due to the presence of certain specific cell wall degrading enzymes such as chitinases, glucananses, proteinases, xylanases, etc. to name a few. Each enzyme has its own role in mycoparasitism thus, it becomes important to study the activity of such enzymes produced by *Trichoderma* species. The induction of xylanase from *T. harzianum* (*Th* Azad) by using different carbon sources was studied by Singh et al. (2012b). Highest xylanase activity (5.8 ±0.01 IU/ml) was observed at 120 h with 1% birchwood xylan as a carbon source.

The study also includes the behavior of these BCAs

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against fungal wilt pathogens affecting leguminous crops (lentil, pigeonpea and chickpea). The strains distribution in several genotypes could also support the idea of developing antifungal formulations in which different *Trichoderma* BCAs could be combined. 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 (Singh et al., 2011; Shahid et al., 2011). *Trichoderma* species, when grown either in Potato dextrose agar (PDA) or Potato dextrose broth (PDB) within a pH range of 7 to 7.5 and at an optimum temperature range of 25 to 30°C gives the best growth and sporulation rates both.

Talc-based bioformulation of *Trichoderma* (Shahid et al., 2012c) 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 METHODS

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 phythopathogens. The most promising isolates were selected for biochemical, molecular and disease suppressiveness tests. Initially, a total of 7 strains were identified and were selected for further study. Based on the descriptions of Bissett (1991), these fungi are classified 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* and *Fusarium oxysporum* that were previously isolated and identified in the Bio-Control 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 Petri dishes containing Malt agar (MA) (Difco). Isolates, which showed a marked effect towards pathogens were selected and used for further study. Each *Trichoderma* isolate was separately inoculated into 100 ml PDB 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 Petri dishes 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 Petri dishes 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.

Xylanase enzyme activity assay

Xylanase enzyme activity was assayed using 1% (w/v) birchwood xylan as a substrate. The reaction mixture contained 1ml of 1% xylan solution (in 0.1M, pH 5 sodium citrate buffer) and 2 ml of enzyme was added to the reaction tubes and incubated at 40°C. The amount of reducing sugar in the reaction tubes was measured using the DiNitrosalicylic Acid Method. After heating the tubes for 5 minutes in a water bath and then cooling, the absorbance was read at 550 nm using UV spectrophotometer. The amount of reducing sugar was calculated from the standard curve based on the equivalent xylose. One unit of xylanase activity is defined as 1 μmol of xylose equivalent produced/ min under the assay conditions.

Simple sequence repeats (SSR) analysis

DNA was extracted using cetyltrimethyl ammonium bromide (CTAB) method from all seven isolates and quantified using agarose gel electrophoresis. SSR primers were selected and polymerase hain reaction (PCR) was programmed with an initial denaturing for 4 min at 94°C; followed by 35 cycles of denaturation for 1 min at 94°C; annealing at 36°C for 1 min; extension for 90 s at 70°C, and a final extension for 7 min at 72°C in a Primus 96 advanced gradient Thermocycler. PCR product (20 μl) was mixed with loading buffer (8 μl) 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 (2001) in 2% agarose in Tris-acetate-EDTA (TAE) buffer (for a litre of 50X TAE Stock solution, 242 g Tris Base, 57.1 ml Glacial Acetic Acid and 100 ml 0.5M EDTA was used), stained with 0.2 μg/ml ethidium bromide. Nucleic acid bands were photographed and detected by BioRad Gel Doc system.

Mass multiplication of *Trichoderma harzianum (Th.azad)* strain

Bajra (Pennisetum typhoides) grains should be completely soaked in 2% sucrose solution in water for 6 h. After draining out the excess water the soaked 250 g seeds of bajra should be filled in autoclavable poly-propylene (PP) bags of 30 × 20 cm^2. The PP bags should be plugged with nonabsorbent cotton followed by autoclaving at 15 lbs pressure for 30 min. After autoclaving, the bags should be left for cooling overnight. Next day the bags should be individually inoculated by using 5 ml stock solution (10^6 - 10^8 CFU/ml) of starter culture grown for 100 days, with syringe. Before inoculation, the place from where the inoculation is to be made should be marked out with a small circle with the help of marker pen. Punctured place of injection of the PP bag must be sealed with cellophane tape. The bags should be incubated at 25 ± 2°C for 15 days in a temperature controlled room. After 15 days of incubation, the contents of the bags should be taken out and kept in hot air oven for drying overnight at 35°C. During the 15 days of incubation visual check every day is essential to ensure detection and elimination of contaminated PP bag(s). Formulation thus prepared should be ground to fine powder, while ensuring that during the process temperature does not go beyond at 35°C. The powdered formulation thus obtained should be mixed with pre-sterilized talc in 1:9 (Trichoderma spore:talc) ratio. Three samples should be taken from each but lot during production and tested using a standardized method to determine the viability of the active ingredient expressed as colony forming units (CFU). The product thus prepared is ready for packaging at this stage. For storage the finished product should be stored in vacuum filled plastic bags, covered by paper cartons of different sizes (250, 500 and 1000 g). These packets should be

Table 1. Details of *Trichoderma* species.

Strain No.	Name of Bio-agent	Strain code	ITCC Acc. No	NBAIM Acc. No.	Gen bank NCBI No.	Source	GPS
T1	*T. viride*	01PP	8315	F-03110	JX119211	Hardoi (U.P., India)	Latitude: 27˚ 23′ 40.729″ Longitude: 80˚ 7′ 47.751″
T2	*T. harzianum*	*Th* azad	6796	F-03109	KC800922	CSA Kanpur Nagar(U.P., India)	Latitude: 25˚ 8′ 34.821″ Longitude: 81˚ 59′ 2.979″
T3	*T. asperellum*	Tasp/CSAU	8940	F-03108	KC800921	CSA Kanpur Nagar (U.P., India)	Latitude: 26˚ 29′ 33.384″ Longitude: 80˚ 18′ 6.518″
T4	*T. koningii*	T_K (CSAU)	5201	F-03112	KC800923	CSA Kanpur Nagar(U.P., India)	Latitude: 26˚ 29′ 33.384″ Longitude: 80˚ 18′ 6.518″
T5	*T. atroviride*	71 L	7445	F-03107	KC 008065	Hardoi (U.P., India)	Latitude: 26˚ 34′ 27.61″ Longitude: 79˚ 18′ 24.623″
T6	*T. longibrachiatum*	21 PP	7437	F-03111	JX978542	Kaushambi (U.P., India)	Latitude: 25˚ 21′ 39.794″ Longitude: 81˚ 24′ 11.414″
T7	*T. virens*	*T.vi* (CSAU)	4177	F-03106	KC800924	CSA Kanpur Nagar (U.P., India)	Latitude: 26˚ 29′ 28.323″ Longitude: 80˚ 18′ 26.361″

then kept in sealed cartons for transportation purpose (Figure 1).

RESULTS AND DISCUSSION

Isolation and bioassay

Seven isolates of *Trichoderma* sp. were isolated from the soils of pulse fields of various districts of Uttar Pradesh, India. These include *T. harzianum, Trichoderma viride*, *Trichoderma asperellum, Trichoderma koningii, Trichoderma atroviride*, *T. longibrachiatum* and *Trichoderma virens*. All tested strains of genus *Trichoderma* had high or moderate antagonistic activity towards pathogens expressed as a zone of inhibition and fungal growth reduction by using culture filtrate. It was also found that the most effective species were *T. harzianum* and *T. viride* against all test pathogens. Isolated *Trichoderma* strains were submitted to the Indian Type Culture Collection (ITCC) and GenBank (NCBI) database and accession numbers allotted to specific strain of each species (Table 1).

Morphological description

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

Xylanase enzyme production

The growing importance of many *Trichoderma* strains as BCAs and producers of valuable metabolites and enzymes have made their distinction from other *Trichoderma* isolates

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

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	Chlamy-dospores
T. viride (01 PP)	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 (*Th* Azad)	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 (*Tasp* /CSAU)	5 - 6 in 3 days	Snow white green	Orange	Smooth	Floccose	Watery White	Ring like zones	Branched, regular	Smooth	Green	Not observed
T. koningii (*Tk* (CSAU)	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 (71L)	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 (21PP)	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 (*Tvi* (CSAU)	8 - 9 in 3 days	Snow white	Colorless	Smooth	Floccose to Arachnoid	Watery White	Flat	Highly branched, regular	Smooth	Dirty Green	Not observed

essential. *Trichoderma*, being a saprophyte adapted to thrive in diverse situations, produces a wide array of enzymes. Strains of *Trichoderma* can produce extracellular enzymes and antifungal antibiotics that may act as competitors to fungal pathogens and induce resistance in plants. By selecting strains that produce a particular kind of enzyme, and culturing these in suspension, industrial quantities of enzymes can be produced.

Mycoparasitism involving lytic enzymes has been described as the mechanism of action of *Trichoderma* species in the biological control of commercially important plant pathogens. However, little information is available on the significance of this mechanism for the biological control of soil borne pathogens. Xylanase enzyme activity for all seven *Trichoderma* isolates on broth media containing different carbon sources is shown in Figure 1. Maximum activity of xylanase on media containing birchwood xylan was found in *T. harzianum* (*Th* Azad) followed by *T. viride* 01PP, *T. asperellum* (*Tasp*/ CSAU), *T. koningii* (*Tk*/(CSAU), *T. atroviride* (71 L), *T. longibrachiatum* (21PP) *and T. virens* (*Tvi* (CSAU) etc.

The objective of the study is to extract and characterize xylanases from *Trichoderma* sp. Isolated from environmental soil samples. The metabolic activity and enzyme productivity of fungi is influenced by environmental conditions such as pH, cultivation period and temperature, concentration and the nature of the substrate used. In this context, the aim is to analyze various factors that may increase the xylanase production in the fungus *Trichoderma* such as, initial pH of the growth environment, carbon source, cultivation period and concentration of substrate used in the growth environment.

For commercial realization and economic viability of xylanase production, it is necessary to identify organism which can hyper-produce the enzymes. Some physiological conditions like the

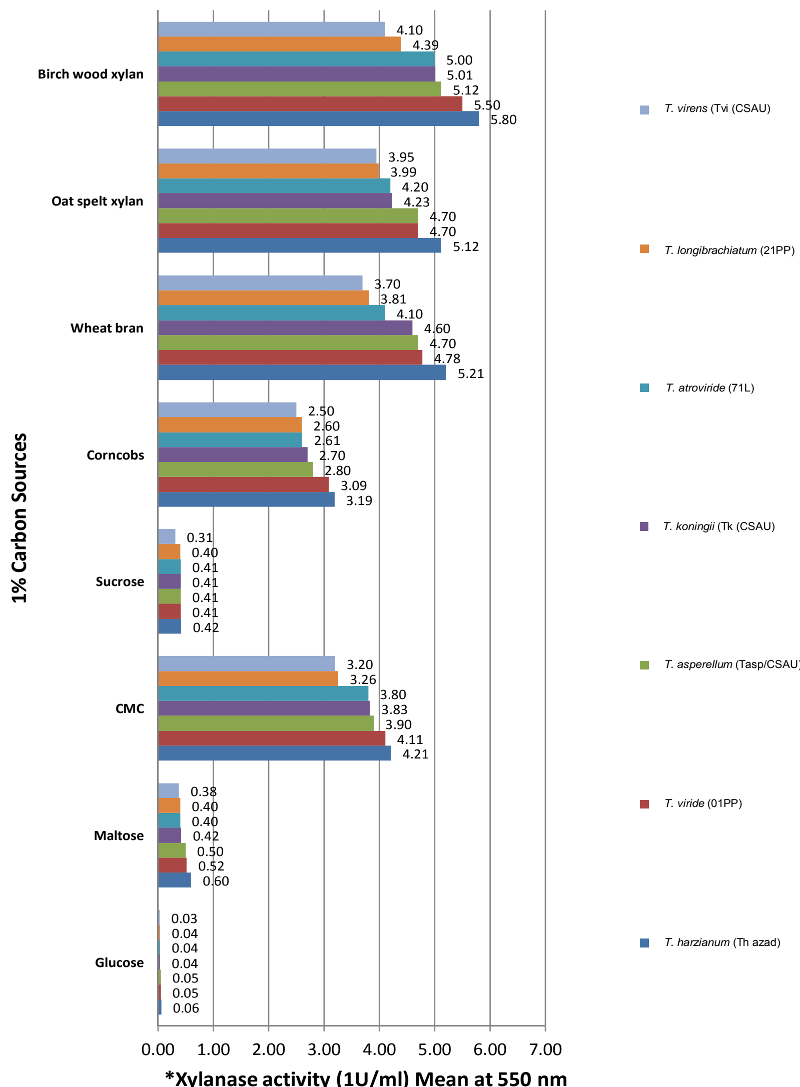

Figure 1. Xylanase activity produced by *Trichoderma spp.* grown on different carbon sources.

activity of xylanase enzyme produced from *Trichoderma* sp. by using different carbon sources, pH, temperature and substrate concentration were determined (Table 3).

Molecular characterization of Trichoderma sp. using SSR markers

Set of SSR primers were used. The preliminary studies indicates that *Trichoderma* isolates has 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 Unweighted pair-groups method using arithmetic averages (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 seven strains of *Trichoderma* sp. 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. This would enable us to develop a potential strain possessing competitive ability, growth promoting characters and inducing resistance in plants.

Dendrogram shown in Figure 2 revealed that all the

Table 3. Properties of fungal xylanase.

Organism	Name of strains	Optimum pH	Optimum temperature (˚C)
T. viride	01PP	4.5	55 - 60
T. harzianum	*Th* Azad	5.0	45
T. asperellum	*Tasp* /CSAU	4.0 - 5.0	70
T. koningii	*Tk* (CSAU)	5.0	40
T. atroviride	71L	5.0	60
T. longibrachiatum	21PP	6.0	55
T. virens	*Tvi* (CSAU)	5-5.5	45

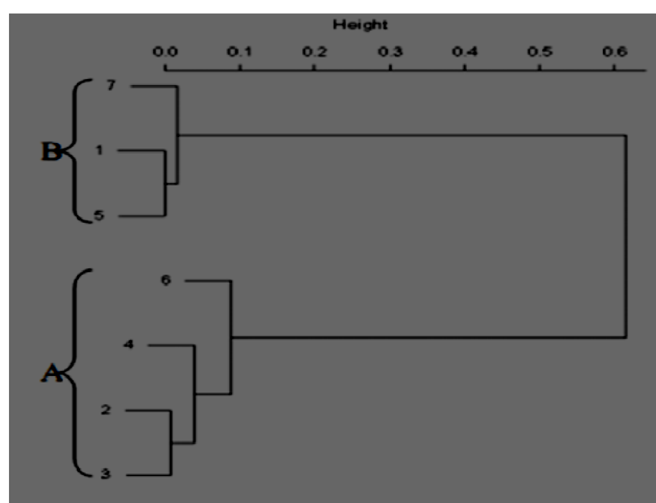

Figure 2. Dendrogram for *Trichoderma spp.* isolates as revealed by SSR markers; where: 1: *Th* Azad, 2: 71L, 3: 21PP, 4: *Tk* (CSAU), 5: *Tasp/*CSAU, 6: *Tvi* (CSAU), 7: 01PP.

seven isolates of *Trichoderma* sp. were distinctly divided into two major Clusters A and B at 20 units. Isolate *Tk*/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 *Tasp*/CSAU and *Tk* (CSAU). Isolates *Tk* (CSAU), 71L, 21PP, and *Tvi* (CSAU) were assigned to Cluster 'A'. Genetic dissimilarity among the entries in this cluster ranged from a lowest of 14.3% (between 21PP and *Tvi* (CSAU) to a highest of 35.7% (between *Tvi* (CSAU) and *Tk* (CSAU). The other Cluster 'B' comprised of three isolates namely *Th* Azad, *Tasp* (CSAU) and 01PP were grouped together. The genetic dissimilarity in this group ranged from 33.3% between *Tasp* (CSAU) and 01PP to 75% between *Th* Azad and 01PP (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 bio-efficacy simultaneously.

Shelf life of *Trichoderma* in talc as a carrier material was determined 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. That is, 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 4).

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. harzianum* (*Th* Azad) 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

Table 4. A matrix displaying the genetic distances between the seven isolates of *Trichoderma* sp.

Strain	*Th* Azad	71L	21PP	*Tk*/CSAU	*Tasp*/CSAU	*Tvi* (CSAU)	01PP
Th Azad	0						
71L	0.544	0					
21PP	0.643	0.231	0				
Tk/CSAU	0.544	0.333	0.231	0			
Tasp/CSAU	0.667	0.769	0.643	0.857	0		
Tvi (CSAU)	0.643	0.231	0.143	0.357	0.643	0	
01PP	0.750	0.643	0.533	0.733	0.333	0.533	0

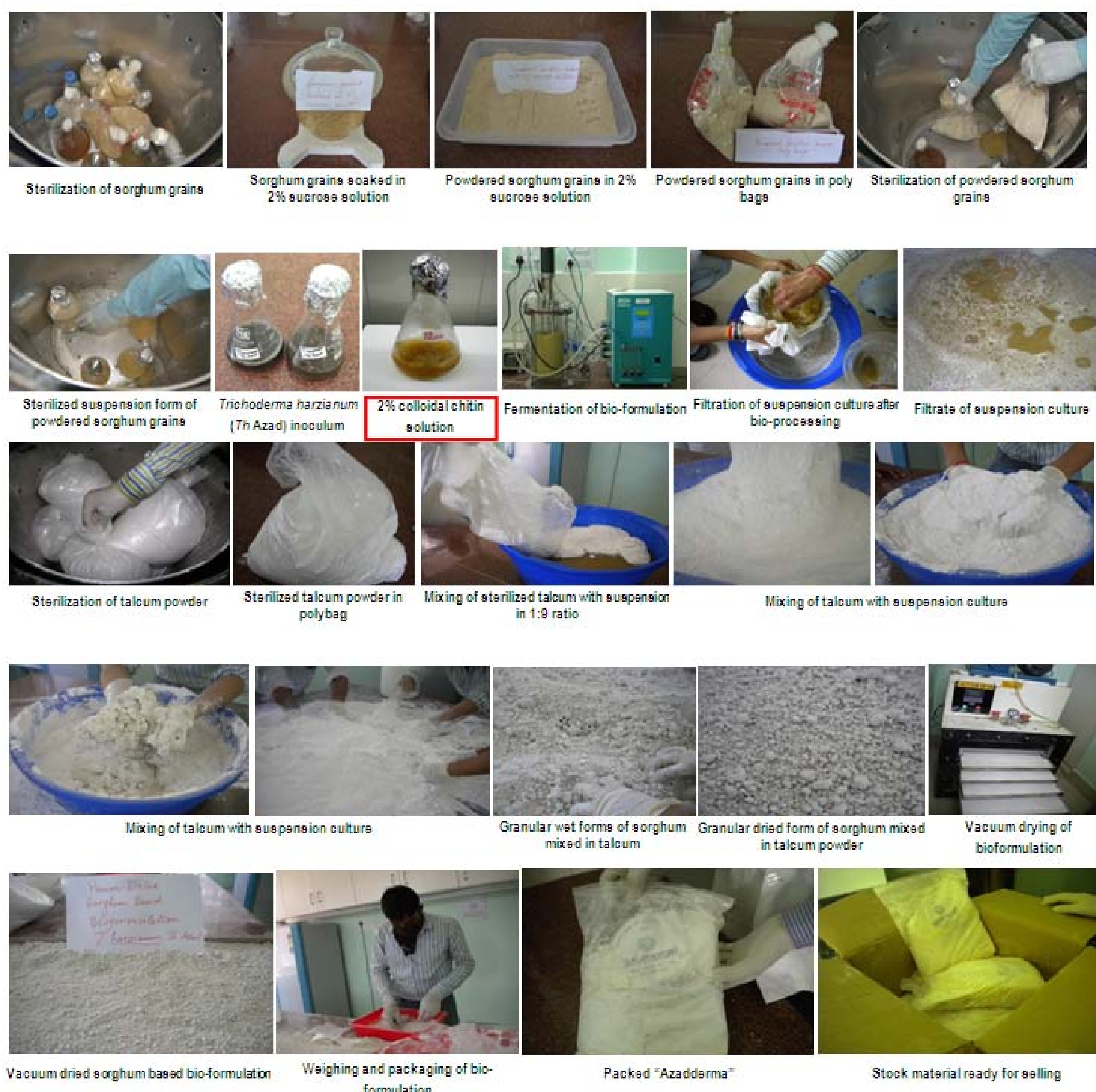

Figure 3. Production steps of *Trichoderma harzianum* (Th Azad) bioformulation.

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.

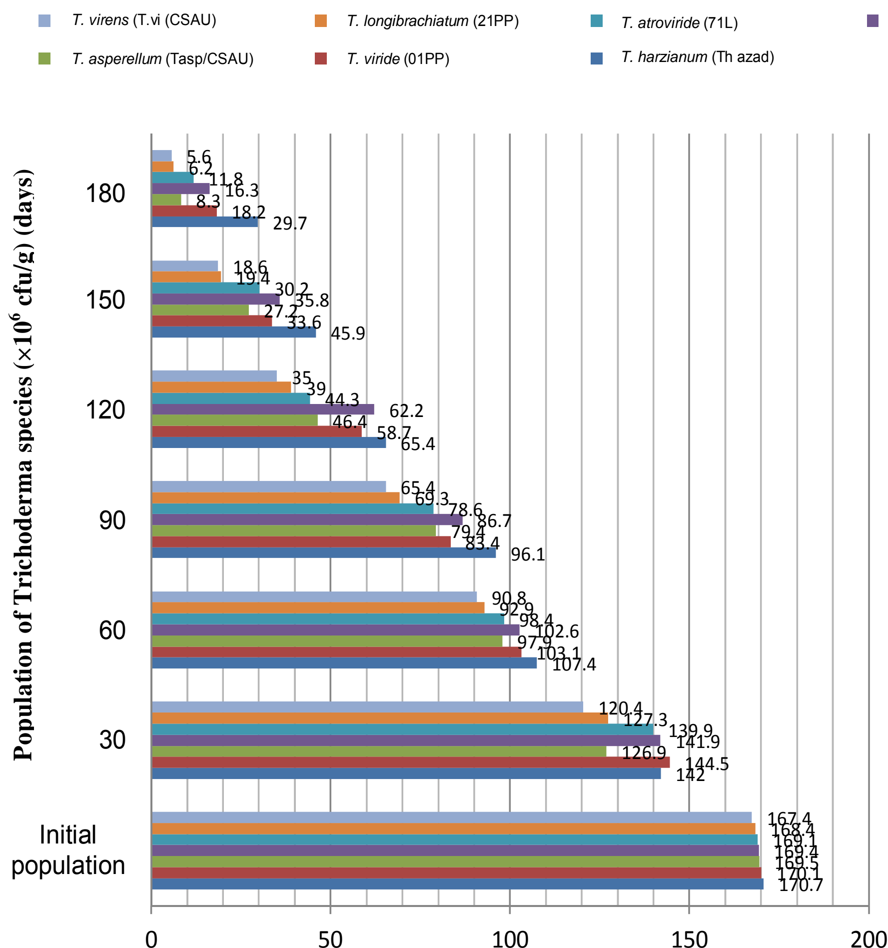

Figure 4. Effect of Talc as a carrier on the population of *Trichoderma* spp.

Table 5. Effect of bioagent *Trichoderma harzianum* (*Th* Azad) on seed germination, root and shoot length and seedling vigour in chickpea (Paper Towel Method).

Parameter	Treated Seeds	Control
Seed Germination	87.66%	80.56%
Root Length	9.7 cm	8.8 cm
Shoot Length	5.2 cm	4.9 cm
Vigour Index*	1306.13	1103.67

Note: The data on the percent seed germination, root and shoot length and seedling vigour index recorded after seven and ten days of sowing, respectively.

The efficacy of bioformulation was ascertained on the seed germination and seedling vigour under laboratory conditions by using paper towel method. It was found that seed treatment with the bioformulation *T. harzianum* (*Th* Azad) (5 g/kg seeds) was able to increase the seed germination, root and shoot length and seedling vigour over untreated ones (Table 5).

Validation of the bioformulation under in vitro conditions

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 pigeonpea

crops. As compared with the control and other strains, application of *T. harzianum* (*Th* Azad) was more effective in reducing the wilt disease caused by *Fusarium. 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 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. harzianum* Rifai 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.

Conclusion

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

Conflict of Interests

The author(s) have not declared any conflict of interests.

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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.

REFERENCES

Harman GE, Howell CR, Viterbo A, Chet I, Lorito M (2004). *Trichoderma* species—opportunistic, avirulent plant symbionts. Nat. Rev. Microbiol. 2:43–56. http://dx.doi.org/10.1038/nrmicro797

Monte E, Llobell A (2003). *Trichoderma* in organic agriculture. V Congreso Mundial del Aguacate pp.725-733.

Bissett J (1991). A revision of the genus *Trichoderma*. II. Infrageneric classification. Canadian J. Bot. 69:2357-2372. http://dx.doi.org/10.1139/b91-297

Gams W, Bissett J (1998). Morphology and identification of *Trichoderma*. and *Gliocladium*. Taylor & Francis, London, UK. pp. 3-34. http://dx.doi.org/10.2307/3761332

Gams W, Meyer W (1998). What exactly is *Trichoderma harzianum*? Mycologia 90:904-915.

Samuels GJ (2006). *Trichoderma*: Systematics, the sexual state, and ecology. Phytopathology 96:195-206. http://dx.doi.org/10.1094/PHYTO-96-0195

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. http://dx.doi.org/10.1631/jzus.2005.B0100

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.

Shahid M, Singh A, Srivastava M, Biswas SK (2012a). 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.

Sagar MSI, Meah MB, Rahman MM, Ghose AK (2011). Determination of genetic variations among different Trichoderma isolates using RAPD marker in Bangladesh. J. Bangladesh Agric. Univ. 9:9-20. http://dx.doi.org/10.3329/jbau.v9i1.8738

Shahid M, Singh A, Srivastava M, Rastogi S, Pathak N (2013). Sequencing of 28SrNA Gene for Identification of *Trichoderma longibrachiatum* 28 CP/ 74444 Species in Soil Sample. Int. J. Biotechnol. for Wellness. Indust. 2:84-90.

Shahid M, Singh A, Srivastava, Rastogi S, Pathak N (2012b). Induction of Xylanase from *Trichoderma viride* by using Different Carbon Sources. Indian J. Agric. Biochem. 25:163-166.

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. Plant. Dis. Sci. 6:32-34.

Shahid M, Singh A, Srivastava, Mishra RP, Biswas (2011). Effect of temperature, pH and media for growth and sporulation of *Trichoderma longibrachiatum* and self life study in carrier based formulations. Ann. Plant. Protec. Sci. 19:147-149.

Shahid M, Singh A, Srivastava M, Pathak N, Rastogi S, Srivastava AK (2012c). Evaluation of Antagonistic activity and Shelf life study of *Trichoderma viride* (01 PP-8315/11). Advan. Life Sci. 1:138-140.

Sambrook J, Russell DW (2001). Agarose Gel Electrophoresis. CSH Protocols.