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# Morphological and Molecular Characterization of *Trichoderma* Isolates: An Antagonist against Soil Borne Pathogens

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**Abstract:** ***Trichoderma has attained importance as a substitute of chemical pesticides all over the world. Hence, an attempt was intended to corroborate the positive relatedness of molecular and morphological characters with antagonistic ability. Among many isolates of Trichoderma atroviride isolated from rhizospheric soils from different parts of U.P. has brought attention due to its highly antagonistic activity. Eight isolates of Trichoderma atroviride were assessed for their mycoparasitic and molecular variability. These were isolated on PDA medium by serial dilution and identified based on phenotypic characters like colony colour, growth, shape of conidiophore, phialides and conidia. Antagonistic variability of the isolates of T. atroviride revealed significant suppression in the radial growth of Fusarium oxysporum f. sp. ciceri, F. oxysporum f. sp. lentis and F. oxysporum f. sp. udum. Maximum inhibition (60.26%) of mycelial growth was recorded in case of $TH_1$ isolate against Fusarium oxysporum f. sp. lentis (F.o.l.) which was isolated from the soil sample of Bilgram (Hardoi), followed by $TE_8$ (50%) isolated from Bharthana (Etawah). $TS_5$ (39.76%) isolate from Lainbua (Sultanpur) was found to be least effective against F.o.l. Similarly, in case of Fusarium oxysporum f. sp. ciceri (F.o.c), the maximum inhibition (55.08%) of mycelial growth was shown by the isolate $TH_3$, which was isolated from the soil sample of Bilgram (Hardoi). The next effective isolate was $TS_6$ (53.65%) isolated from Kadipur (Sultanpur). Isolate $TSi_4$ (37.69%) of Misrikh (Sitapur) was found least effective against F.o.c. Maximum inhibition in case of Fusarium oxysporum f. sp. udum (F.o.u) was recorded with isolate $TA_{U8}$ (47.91%) from Azeetmal (Auraiya). Next effective isolate was $TB_{a1}$ (47.25%) isolated from Ballaha (Bahraich). The least effective isolates were $TS_{i3}$ (41.66%) and $TKD_3$ (41.66%) isolated from Misrikh (Sitapur) and Maitha (Kanpur Dehat). Molecular variability among the isolates showed 74 amplified bands out of which 65 were polymorphic and 19 were monomorphic. The size of amplified product varied from 0.1kb to 0.75kb.***

**Keywords:** Trichoderma atroviride, antagonism, morphological and molecular character, DNA extraction, RAPD

## 1. Introduction

Plant disease epidemics have created an ecologically imbalance system in modern agriculture. Deterrence of such epidemics for the most part achieved through the use of chemical fungicides has greater repercussion on environment and human health. Also, progressive confrontation in a midst of pathogen resistance to accessible chemical plant protectants has engrossed the need of alternative methods of disease control. Fungi of the genus *Trichoderma* are important biocontrol agent against several soil borne phytopathogens (Benitez *et.al*.2004). *Trichoderma* spp. are free living rapid growing fungi that are common in soil and root eco-system. The fungi are exceptionally good model for biocontrol more importantly as bioagent, which is accomplished by means of mycoparasitism, antibiosis and competition. Several species of *Trichoderma* have been isolated from various substrates and locations (Bilgrami *et.al.*1971, Nagamani *et.al.* 2002). Several articles have also been published to identify the *Trichoderma* spp. based on the molecular or physiological bases (Samuels, 1996, Hermosa *et.al*. 2000). Kiffer and Morelet (2000) have recognized several species of *Trichoderma* based on molecular characters. The molecular techniques like Random Amplified Polymorphic DNA (RAPD) developed by Williams *et al.* (1990) has been used for genetic and taxonomic studies for several fungi including *Trichoderma* sp. (Muthumeenashi and Mills, 2004). Most of the species of *Trichoderma* are effective against soil borne pathogens that cause diseases in leguminous crop. Fusarium wilt is the most important disease that significantly damages the crop. A high level of genetic diversity in *Trichoderma* spp. has been reported (Chakraborty et.al. 2010) that can be used to produce a wide range of products of commercial and ecological interest.

## 2. Materials and Methods

### 2.1 Identification and Morphological characterization of *Trichoderma sp.*

Isolates of *Trichoderma* were isolated from soil samples collected from rhizospheric of chickpea, pigeonpea and lentil crop from different places of Uttar Pradesh, India. All the isolates were isolated on PDA medium by following serial dilution plate technique as described by Johnson and Curl [8] and isolates were identified up to species level based on phenotypic characters like colony colour and growth; size and shape of conidiophore, phialides and conidia. The cultures were identified using the available literature [9-12] and confirmed by morphological characters and also confirmed by ITCC, Division of Plant Pathology IARI, New Delhi-12. The morphological characterization was based on monographic contribution provided by Bissett (1991 a, b).

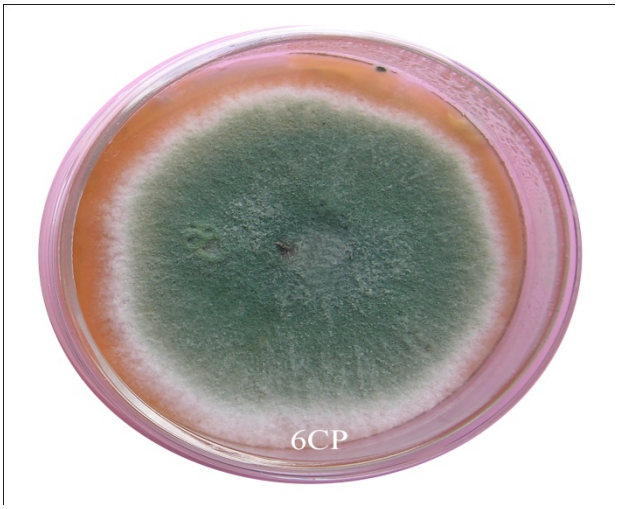
### 2.2 Confrontation assays *in vitro*

The antagonistic potentiality of the isolates of *T. atroviride* was determined against three strains of *Fusarium* by dual culture technique described by Morton and Stroube (1955). A disc of 5 mm diameter was made from 7 days old culture of different isolates of *T. atroviride* and placed at one point leaving 1 cm distance from the periphery of one side of Petri plate and on the opposite site, disc (5 mm dia.) of F.o.u, *F.o.c* and *F.o.l* were placed separately. Plate was kept without antagonist served as control. These Petri plates were incubated at 23 ± $2^0$C for 7 days in three replications (Table 3). Observations on colony growth were recorded and percent inhibition was measured by using the following formula:-

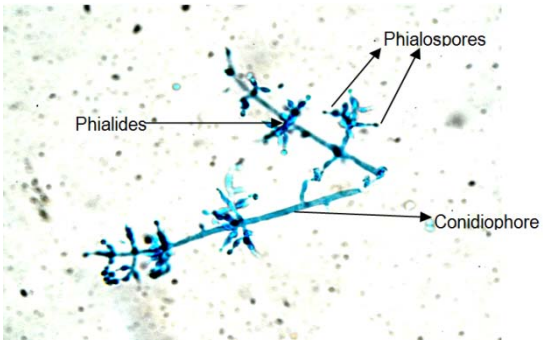
$$I=\frac{(C-T)}{C}\times 100$$

### 2.3 Molecular characterization

Genomic DNA was extracted from each isolates of *Trichoderma atroviride* grown in 1000 ml conical flask containing 400 ml of PDB medium. Two agar plugs from actively growing colony of *T. atroviride* were transferred to each flask aseptically in a laminar flow. The flask was incubated at 23±$2^0$C for 7 days. The mycelial mat was collected by passing the fluid through three layers cheese cloth. The fungal cell wall was disrupted by grinding with pestle and mortar in liquid nitrogen. The DNA was extracted by CTAB method of fungal DNA extraction as used by Kumar *et al.* (2011). Quantification of DNA was done with 0.8% agarose gel electrophoresis. Working concentration of DNA was adjusted to 20 ng/µl and stored at $4^0$C. The DNA from all isolates produced clear and sharp bands, indicating good quality of DNA.




**(a)**




**(b)**

**Figure 1:** *Trichoderma atroviride*: (a) Growth on PDA media, (b) Microscopic observation at 100x

### 2.4 Random Amplified Polymorphic DNA

The procedure described by Williams *et al.* with minor modification like in PCR cycles standardization was done for carrying out PCR reaction to produce RAPD profiles. Amplification of DNA fragments was carried out by the PCR using 10-mer arbitrary primers. The reaction mixture consisted of 300ng of 200 µmM of dNTP mix (Fermentas company), 15 pmol of primer (Operon), 5 U/µl of Taq polymerase (Fermentas) and 25 mM $MgCl_2$. DNA amplifications were performed in thermocycler with one cycle of initial denaturation at 94°C for 15 min, followed by 40 cycles of denaturation at $94^0$C for 1 min, annealing at 35°C for 2 min, extension at 72°C for 1 min and final extension at 72°C for 10 min. Amplified products together with uncut lambda marker double digest; Bangalore, Genei) were resolved by 1.5% agarose gel electrophoresis (60 V for 1 hr). Gels were photographed by (Uvitec) Gel documentation system (Figure 1).

### 2.5 Data Analysis

Comparison of each profile for each primer was done on the basis of the presence or absence (1/0) of amplified bands. Bands of the same size (in bp) were scored as identical. All reproducible polymorphic bands were scored and analysed following UPGMA cluster matrix using NTSYSpc (Numerical Taxonomy System Biostatistics, version 2.02e).

## 3. Results and Discussion

### 3.1 Morphological characterization

The growth patterns of *Trichoderma* isolates after four days of incubation at 23 ± $2^0$C on PDA media showed significant differences in nature of culture growth and sporulation patterns. The colony colour changes from light green shade to dull green with the production of conidia. The conidial wall patterns and shape were rough, subglobose and smooth (Fig.1). The growth characters of culture and sporulation patterns varied noticeably within and between the isolates (Table 2).

**Table 1:** Key morphological description used for characterization of *T.* isolates

| Isolate Codes/ Characters | 6 CP | 24 CP | 71 L | 115 L | 52 L | 75 PP | 126 PP | 105 CP |
|---|---|---|---|---|---|---|---|---|
| Colony growth rate (cm/day) | 7-8 in 5 days | 7-8 in 5 days | 6-7 in 5 days | 7-8 in 5 days | 7-8 in 5 days | 7-8 in 5 days | 7-8 in 5 days | 6-7 in 5 days |
| Colony colour | Light Green to yellowish green | Light Green to dark green | Light Green to dark green | Light Green to yellowish green | Light Green | Light Green to dark green | Light Green to yellowish green | Light Green to dark green |
| Reverse colony colour | Yellow | Light yellow | Colourless | Light yellow | Yellow | Light yellow | Colourless | Light yellow |
| Colony edge | Smooth | Smooth | Smooth | Smooth | Smooth | Smooth | Smooth | Smooth |
| Culture smell | Malt | Malt | Malt | Malt | Malt | Malt | Malt | Malt |
| Mycelial form | Arachnoid | Floccose to arachnoid | Floccose to arachnoid | Floccose to arachnoid | Arachnoid | Arachnoid | Arachnoid | Floccose to arachnoid |
| Conidiation | Crusty in surface | Crusty in surface | Crusty in surface | Crusty in surface | Crusty in surface | Crusty in surface | Crusty in surface | Crusty in surface |
| Phialide shape | Ampulliform | Ampulliform | Oblong | Ampulliform | Oblong | Ampulliform | Ampulliform | Ampulliform |
| Conidial shape | Subglobose & Smooth | Subglobose , Smooth | Subglobose, Smooth | Subglobose, Smooth | Subglobos, Smooth | Subglobose, Smooth | Subglobose, Smooth | Subglobose, Smooth |
| Conidial wall | Rough | Rough | Rough | Rough | Rough | Rough | Rough | Rough |
| Conidial colour | Yellowish green | Yellowish green | Green | Yellowish green | Yellowish green | Green | Green | Yellowish green |
| Chlamydosopores | Not observed | Not observed | Present globose | Not observed | Not observed | Not observed | Not observed | Not observed |

### 3.2 Confrontation assays in vitro

The isolates of *Trichoderma viz.* 6 CP, 24 CP, 71 L, 115 L, 52 L, 75 PP, 126 PP and 105 CP belong to *T. atroviride.* In order to evaluate the antagonistic potential of *T. atroviride*, dual culture technique was carried out with three legume pathogens *viz. F. oxysporum* f. sp. *ciceri*, *F. oxysporum* f. sp. *lentis* and *F. oxysporum* f. sp. *udum*. Effects of different isolates of T. atroviride with respect to suppression of mycelial growth of the three test pathogens were recorded. It is evident from the data that *T. atroviride* suppressed the radial growth of *F. oxysporum ciceri, F. oxysporum lentis* and *F. oxysporum udum* significantly (Table 3). Maximum inhibition (60.26%) by *T. atroviride* $TH_1$, isolate, which was isolated from soil sample of Bilgram block of Hardoi district, followed by $TE_8$ (50.00%) isolated from Bharthana block of Etawah district. $TS_5$ (39.76%) isolate of Lainbua block of Sultanpur was found least effective against the pathogen. The findings clearly show the wide range of antagonistic effect of different isolates of *T. atroviride.* The maximum inhibition (47.9%) of mycelial growth of *F. udum* was recorded in case of $TAU_8$ isolate, which is isolated from the soil sample of Azeetmal block of Auraiya. $TSI_3$ and $TKD_3$ isolate of Misrikh and Maitha were found to be least effective against *F. udum* among all the isolates of *T. atroviride.* Joshi *et al*. (2010) also found the antagonistic variability in different isolates of *Trichoderma* spp. collected from different places of India. The present finding was also supported by several workers (Obaiua Oti, 2007). Singh *et al*. (2013) also revealed that 30 isolates of *Trichoderma viride* collected from various districts of U.P. were found highly antagonist against three test pathogens *(F.o.u, F.o.c* and *F.o.l*).

**Table 2:** *In vitro* evaluation of different isolates of *T. isolates* against soil borne pathogens

| I.D. No. | Culture No. | % inhibition of *Trichoderma* isolates against *F. oxysporum* f. sp. *udum* | % inhibition of *Trichoderma* isolates against *F. oxysporum* f. sp. *ciceri* | % inhibition of *Trichoderma* isolates against *F. oxysporum* f. sp. *lentis* | % inhibition of *Trichoderma* isolates against *S. rolfsii* |
|---|---|---|---|---|---|
| ITCC-7442/09 | 06 CP | 45.83 | 53.65 | 39.76 | 54.83 |
| ITCC-7443/09 | 24CP | 41.66 | 37.69 | 44.88 | 58.03 |
| ITCC-7445/09 | 71L | 45.83 | 55.08 | 60.26 | 62.26 |
| ITCC-7446/09 | 115L | 47.25 | 52.17 | 42.30 | 45.16 |
| ITCC-7447/09 | 52L | 43.75 | 40.00 | 43.07 | 41.93 |
| ITCC-7448/09 | 75PP | 47.91 | 43.47 | 48.07 | 51.61 |
| ITCC-7449/09 | 126PP | 41.66 | 43.43 | 46.92 | 55.08 |
| ITCC-7451/09 | 105PP | 45.83 | 43.91 | 50.00 | 52.00 |
| CD at 5 % | | 2.71 | 2.52 | 2.93 | 1.06 |
| SE | | 1.28 | 1.19 | 1.38 | |

### 3.2 Molecular Characterization

The results presented in Fig. 2 showed that the total number of reproducible bands amplified were 94, out of which 75 were found to be polymorphic and 19 were monomorphic, hence the percentage of polymorphism is 79.78. The number of bands per primer ranged from the maximum of 12 (given by OPC-13) to a minimum of 3 (OPC-8) with an average of 7 bands per primer.

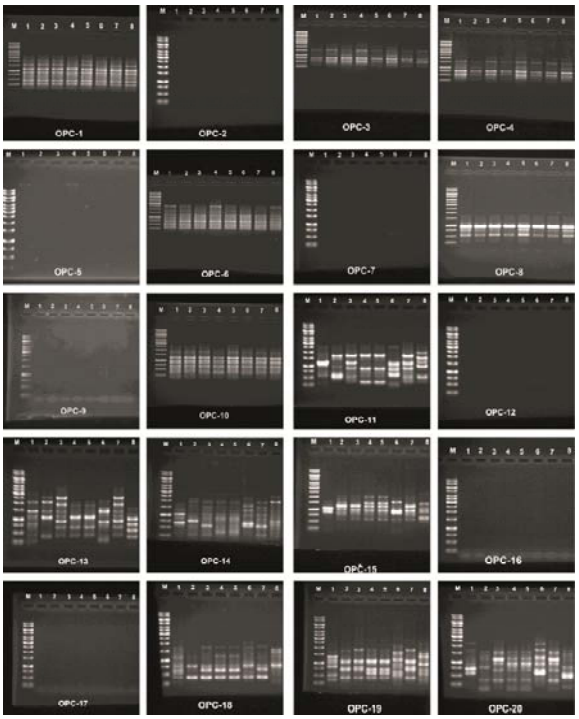



**Figure 2:** RAPD analysis of *T. atroviride* isolates with 20 primers (OPC-1-OPC-20)

The experimental findings also revealed that the 7 RAPD primers (OPC 10, 11, 13, 14, 15, 19 & 20) produced average or above average amplified products (Table 4).

**Table 3:** RAPD amplification and their corresponding PCR products for *T. atroviride*

| *Name of Primer OPC* | *Sequence of Primer 5′ – 3′* | *No of polymorphic band* | *No of Monomorphic bands* | *Total no. of Bands* |
|---|---|---|---|---|
| OPC 1. | TTCGAGCCAG | 2 | 4 | 6 |
| OPC 2. | GTGAGGCGTC | 0 | 0 | 0 |
| OPC 3. | GGGGGTCTTT | 3 | 1 | 4 |
| OPC 4. | CCGCATCTAC | 3 | 1 | 4 |
| OPC 5. | GATGACCGCC | 0 | 0 | 0 |
| OPC 6. | GAACCGACTC | 3 | 3 | 6 |
| OPC 7. | GTCCCGACGA | 0 | 0 | 0 |
| OPC 8. | TGGACCGCTG | 1 | 2 | 3 |
| OPC 9. | CTCACCGTCC | 0 | 0 | 0 |
| OPC 10. | TGTCTGGGTG | 2 | 5 | 7 |
| OPC 11. | AAAGCTGCGG | 10 | 0 | 0 |
| OPC 12. | TGTCATCCCC | 0 | 0 | 0 |
| OPC 13. | AAGCCTCGTC | 11 | 1 | 12 |
| OPC 14. | TGCGTGCTTG | 11 | 0 | 11 |
| OPC 15. | GACGGATCAG | 7 | 0 | 7 |
| OPC 16. | CACACTCCAG | 0 | 0 | 0 |
| OPC 17. | TTCCCCCCAG | 0 | 0 | 0 |
| OPC 18. | TGAGTGGGTG | 3 | 2 | 5 |
| OPC 19. | GTTGCCAGCC | 0 | 0 | 0 |
| OPC 20. | ACTTCGCCAC | 9 | 0 | 9 |
| **GRAND TOTAL** | | **75** | **19** | **94** |

The size of the amplified product varied from minimum of 0.1 kb to maximum of 750 bp i.e., 0.75 kb. Thus, the presence or absence of bands mentioned in Table 2 indicated that the variability existed among the isolates. The relationship among the isolates was evaluated by cluster analysis of the data based on the similarity matrix. The dendrogram was generated by Unweighted Pair-Group Method with Arithmetic Mean (UPGMA) in NTSYSpc 2.02e software. It is evident from the dendrogram that the genetic diversity among all eight isolates has been identified by SAHN (Sequential agglomerative hierarchic nonoverlapping) clustering approach that has clustered the isolates into two main groups. The first main group is further subgrouped into two, say, A and B. The isolates that lie in the subgroup A are 6CP, 24CP, 126PP and 105CP whereas, the subgroup B consists of 71L and 115L. The remaining two isolates viz. 75PP and 52L can be considered as the outliers as they do not lie close in relationship to the other six isolates. Chakraborty *et al.* (2010) found the variability based on RAPD analysis among nineteen isolates of *T. viride* and *T. harzianum* obtained from rhizosphere soil of plantation crops, forest soil, and agricultural fields of North Bengal. Pervaiz *et al.* (1999) was also found that in Precise Detection and tracing of *Trichoderma hamatum* 382 in compost amended mixes by using molecular markers.

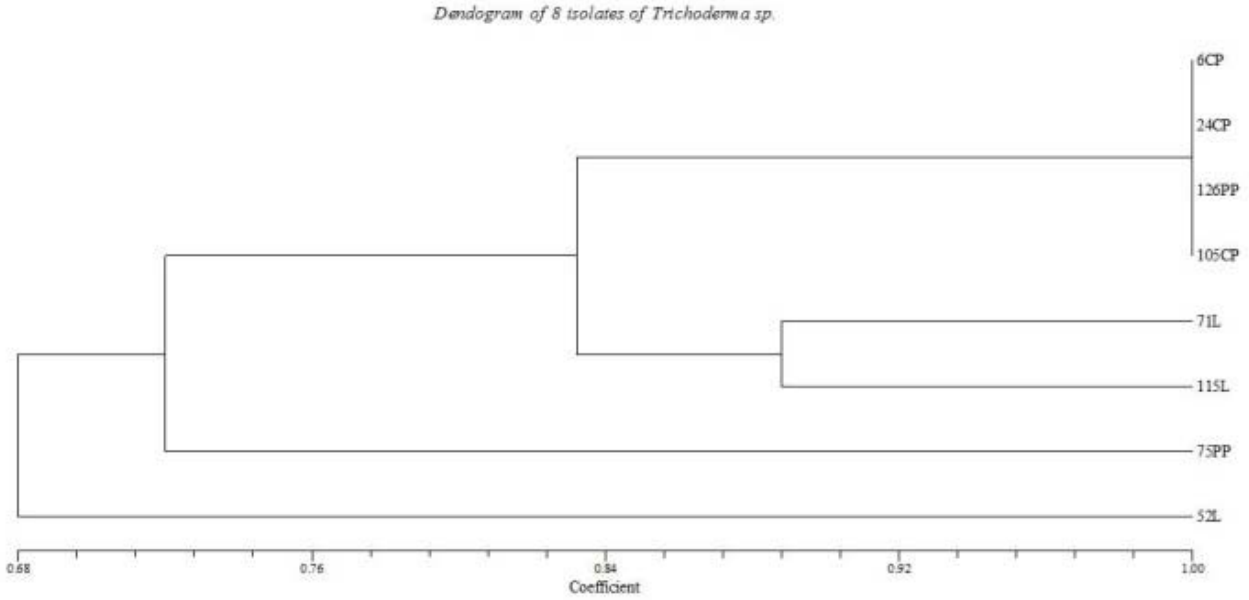



**Figure 3:** Dendrogram showing the genetic relationships among 8 *Trichoderma i*solates based on RAPD analysis

## 4. Conclusion

It may be concluded from the present findings that antagonistic, morphological and molecular variability exist among eight isolates of *T. atroviride,* collected from rhizosphere soil of different places of Uttar Pradesh. It is also concluded that there is good genetic diversity and these are strong possibility to get the isolates specific primers that will be utilized for particular *Trichoderma* isolates with good biological potential form the field isolates without going the cumbersome bioassay.

## 5. Acknowledgements

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