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# Characterization of *Trichoderma* isolates against *Sclerotium rolfsii*, the collar rot pathogen of *Amorphophallus* – A polyphasic approach

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## HIGHLIGHTS

- Characterization of *Trichoderma* spp. showing varied antagonism to *S. rolfsii*.
- A polyphasic approach include morphological biochemical and molecular attributes.
- Integrated these attributes to correlate with their inhibitory role against *S. rolfsii*.
- *T. harzianum* (Tr9) was selected as the best antagonistic isolate.

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## ABSTRACT

The aim of the present study was to characterize ten selected isolates of *Trichoderma* with differential inhibitory potential over the collar rot pathogen of *Amorphophallus*, *Sclerotium rolfsii*. Use of a polyphasic approach which combined the morphological, molecular and biochemical attributes revealed inter specific and intra specific diversity among different isolates. Strains with the best *in vitro* antagonistic capacity were *Trichoderma harzianum* (Tr9) and *Trichoderma asperellum* (Tr10). In general, relationship was observed between the anatgonistic capacity of the *Trichoderma* spp. and their *in vitro* biocontrol performance, cell wall degrading enzyme production, protein profiles and the RAPD and AFLP finger print. The pot experiment revealed a comparative success of Tr9 in controlling collar rot incidence in *Amorphophallus* over Tr10. This is the first report of intensive characterization of *Trichoderma* strains with the integration of the above data sets against *S. rolfsii*, in an attempt to explore their biocontrol potential in managing collar rot disease.



## 1. Introduction

*Amorphophallus paeonifolius* (Dennst.) Nicolson commonly called as Elephant foot yam is an important tuber crop of tropical and sub-tropical countries which offers an exceptional reach as a cash crop because of its high production potential (50–80 t $ha^{-1}$), market acceptability and lucrative economic returns (Misra, 1997). The crop provides great opportunities in world's food and nutrition security in future as the supply of cereals is decreasing. Elephant foot yam is an excellent and cheap source of carbohydrate and protein, as well as rich in vitamin B6, fiber and omega 3 fatty acids. They are also loaded with potassium, magnesium and phosphorous, as well as with trace minerals like selenium, zinc and copper (Santosa et al., 2014). *Amorphophallus* has been explored for its phytochemical, therapeutic and insecticidal activities. The plant has gained much importance in various pharmacologic potential as analgesic, anti-inflammatory, CNS depressant, antihelmintic, antibacterial, antifungal, antioxidant, antitumour, hepatoprotective, immunomodulatory and cytotoxic agent (Dey et al., 2012; Singh and Wadhwa, 2014).

Among the various field diseases, the collar rot disease caused by *Sclerotium rolfsii* is the most destructive in all *A. paeonifolius* growing areas causing yield loss up to 100% (Misra, 1997). *S. rolfsii* is one of the most devastating soil borne and post harvest pathogen of numerous crop plants particularly of tropics and subtropics, where temperature is adequately high which favor the growth and survival of the fungus (Dasgupta and Mandal, 1989). *S. rolfsii* has an exceptionally broad host range, attacking over 200 genera

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of vegetable, grain, and ornamental crops (Farr et al., 1989). The pathogen has got much economic importance throughout the world due to the large number of sclerotia produced by it and their ability to persist in soil for years thus making it well suited as a facultative parasite (Punja, 1985).

Several methods are employed to combat the disease including the use of chemical fungicides. However, the indiscriminate use of these chemicals has imposed serious environmental impacts (Cook and Baker, 1983). Also, the development resistance in pathogens to these chemicals is another major threat (Dekker and Georgopoulos, 1982). Therefore, developing an eco-friendly approach for managing collar rot is the need of the hour and biocontrol is one such approach. The use of microorganisms as biocontrol agents has provided a very promising alternative and less hazardous method for plant disease control (Cook, 1985). *Trichoderma* spp. represents one of the most widely used biocontrol agents against several economically important plant pathogens.

*Trichoderma* spp. are cosmopolitan and abundant fungi in soil characterized by their rapid growth, capability of utilizing different substrates and resistance to noxious chemicals (Klein and Eveleigh, 1998). The mode of action of *Trichoderma* include inhibition of pathogen by extracellular enzymes (Haran et al., 1996) and antibiotics (Ghisalberti and Rowland, 1993), provide good competition to fungal pathogens (Simon and Sivasithamparam, 1989), promote plant growth (Inbar et al., 1994) and induce systemic resistance in plants (De Meyer et al., 1998). Due to its biological control ability, *Trichoderma* has gained much economic importance and researchers have been working in this genus to explore its novel properties and application in agriculture. The study regarding the behavior of *Trichoderma* is very important and necessary for their effective use since they can act against target organisms in several ways (Jeffries and Young, 1994).

Studies were done to characterize *Trichoderma* species based on their antifungal ability (Haran et al., 1996), phenotypical (Bissett, 1991), biochemical (Grondona et al., 1997), or molecular attributes (Hermosa et al., 2000). Morphological characterization allows the identification of *Trichoderma* at genus level, but the species level is difficult to interpret (Grondona et al., 1997). The incorporation of molecular based analysis was employed for the accurate identification of *Trichoderma* (Lieckfeldt et al., 1998; Druzhinina et al., 2006). Though molecular based analyses are extensively used to study *Trichoderma* sp., not many studies inferred the relationship between morphological, molecular and antagonistic activity data (Hermosa et al., 2000). DNA finger printing methods such as RAPD (Gajera and Vakharia, 2010) and AFLP (Larralde-Corona et al., 2008) analysis were also used to define various *Trichoderma* sp. and discussed the relationship with their antagonistic activity on pathogen. Viterbo et al., 2002 specified the importance of extracellular enzymes and protein analysis to be coupled with *in vitro* confrontation assay as they play vital role in inhibiting plant pathogens.

The present research aims to characterize different isolates of *Trichoderma* with different antagonistic potential against *S. rolfsii*, the collar rot pathogen of *Amorphophallus* using morphological molecular and biochemical approaches. The study also attempts to integrate these attributes to correlate with the inhibitory activity which could be used to select suitable biocontrol agents.

## 2. Materials and methods

### 2.1. Fungal strains

Microorganisms were isolated from soil samples collected from different Elephant foot yam growing areas of India and screened for its antagonistic role against *S. rolfsii* by dual culture method. Only a low proportion of the isolated microbes showed considerable *in vitro* inhibition of the hyphal growth of pathogen and was also evident from the preliminary observations that the most of the fungal isolates which were phenotypically identified under *Trichoderma* yielded the best and sustainable antagonism towards the pathogen in minimum time (data not shown). The ten isolates of *Trichoderma* spp. utilized in this study were selected based on the level of reduction in the radial growth rate of *S. rolfsii* which showed no or low, moderate and high antagonistic activity during an *in vitro* direct confrontation assays. *Amorphophallus* which showed typical symptoms of collar rot were collected from field and the pseudostems were sampled for sclerotia, which were inoculated on PDA plates for the isolation of pure culture of pathogen. The identity of *S. rolfsii* was established by its morphological and molecular characterization. The cultures were kept fresh and viable by periodical transfers on PDA medium under aseptic condition throughout the study.

### 2.2. Phenotypic identification

Qualitative and quantitative morphological characterization of the selected strains of *Trichoderma* was recorded on the basis of 28 parameters which also include some antagonistic criteria as included in Table 1. Phenotypic identification was based on character selection and image comparison was established with the help of the Interactive Key program (http://nt.ars-grin.gov/taxadescriptions/keys/*Trichoderma*Index.cfm). Morphological data were coded on 0–2 scale, where 0 is negative and 2 are predominantly positive. A dendrogram was generated with respect to the morphological features (Nei and Li, 1979) based on the unweighted pair group mean algorithm (UPGMA) using the performed TREECON software package version 1.3 (Van de Peer and Dewachter, 1994). The relative support for the different groups and stability of the dendrogram was assessed by bootstrap analysis (2000 replicates).

### 2.3. DNA extraction and molecular identification

The fungal isolates were grown in potato dextrose broth at 25 °C for 48 h under shaking conditions (100 rpm). The mycelia were collected by filtration and the DNA was extracted from 200 mg of fresh mycelia ground in liquid nitrogen following the method of Sharma et al. (2009). Molecular identification was based on DNA sequencing of ITS1-5.8s-ITS2 ribosomal DNA regions and the *tef*1 gene. The ITS regions were amplified using the universal ITS1 and ITS4 primers (White et al., 1990). PCR amplification was performed in a final volume of 25 µl containing 100 µM (each) deoxynucleoside triphosphate, 0.4 µM of both primers, 2.5 µl of 10× Taq buffer A and 2.5 U of Taq DNA polymerase. PCR was programmed in an Agilent sure cycler 8800 (Agilent technologies, USA) with an initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 51.6 °C for 1 min and extension at 72 °C for 1 min 30 s and the final extension at 72 °C for 8 min. The *tef*1 fragment was amplified by PCR using the same PCR reaction mix mentioned above with the primers ef1 (5′-CATCGAGAAGTTCGAGAAGG-3′) and ef2 (5′-TACTTGA AGGAACCCTTA-3′) (Druzhinina et al., 2004) under the PCR conditions: initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 48 °C for 30 s and extension at 72 °C for 1 min and the final extension at 72 °C for 10 min. Both the ITS and *tef*1 bands were gel purified using Gene JET Gel Extraction kit (Fermentas, EU) and directly sequenced using the primers used for amplification Sequencing was carried out in the DNA fingerprinting wing of Rajiv Gandhi Centre for Biotechnology, RGCB (Thiruvanathapuram, India).

Sequences obtained for each PCR product were processed to remove indels, transformed into consensus sequence using

**Table 1**
Morphological descriptions of *Trichoderma* isolates.

| Characteristics described | | Tr4 | Tr5 | Tr6 | Tr7 | Tr8 | Tr9 | Tr10 | Tr11 | Tr14 | Tr15 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Conidia | Shape | Subglobose to ovoidal | Oblong to a little ellipsoidal | Subglobose to ovoidal | Subglobose to ovoidal | Subglobose to ovoidal | Subglobose to ovoidal | Ellipsoidal | Subglobose to ovoidal | Subglobose to ovoidal | Subglobose to ovoidal |
| | Length (μm) | 3.87 ± 0.15[a] | 4.13 ± 0.31 | 3.87 ± 0.25 | 2.97 ± 0.21 | 3.93 ± 0.15 | 3.13 ± 0.15 | 3.8 ± 0.2 | 4.07 ± 0.25 | 3.93 ± 0.15 | 4 ± 0.2 |
| | Width (μm) | 2.77 ± 0.15 | 2.5 ± 0.26 | 2.77 ± 0.12 | 2.7 ± 0.1 | 3.5 ± 0.1 | 2.60 ± 0.1 | 2.53 ± 0.21 | 3.17 ± 0.15 | 2.97 ± 0.15 | 3.27 ± 0.25 |
| | Ornamentation | Very finely warted | Smooth | Very finely warted | Smooth | Very finely warted | Smooth | Smooth | Very finely warted | Very finely warted | Very finely warted |
| | Pigmentation of conidia | Green | Green | Green | Green | Green | Green | Deep green | Green | Green | Green |
| Conidiophore | Structure of conidiophore | Long central axis with paired branches | Central axis from which single phialides arise | Long central axis with paired branches | Long central axis with paired branches | Long central axis with paired branches | Long central axis with paired branches | Long central axis with paired branches | Long central axis with paired branches | Long central axis with paired branches | Long central axis with paired branches |
| | Sterile hairs | Absent | Absent | Absent | Absent | Absent | Absent | Absent | Absent | Absent | Absent |
| | Fertile hairs | Absent | Absent | Absent | Absent | Absent | Absent | Absent | Absent | Absent | Absent |
| Pustule formation | | Present | Absent | Present | Absent | Present | Absent | Present | Present | Present | Present |
| Phialides | Morphology | Whorls, aggregate | Solitary | Whorls, aggregate | Whorls, aggregate | Whorls, aggregate | Whorls, aggregate | Aggregate | Whorls, aggregate | Whorls, aggregate | Whorls, aggregate |
| | Length (μm) | 7.93 ± 0.25 | 6.8 ± 0.46 | 10.53 ± 0.25 | 4.03 ± 0.25 | 7.97 ± 0.21 | 6.53 ± 0.12 | 7.47 ± 0.31 | 8.43 ± 0.15 | 7.1 ± 0.26 | 8.37 ± 0.15 |
| | Midpoint width (μm) | 3.2 ± 0.1 | 2.83 ± 0.25 | 2.1 ± 0.3 | 2.3 ± 0.1 | 2.97 ± 0.25 | 3.33 ± 0.15 | 2.97 ± 0.21 | 2.9 ± 0.2 | 3.4 ± 0.1 | 3.03 ± .15 |
| | Base width (μm) | 2.03 ± 0.25 | 1.73 ± 0.25 | 2.6 ± 0.26 | 2.13 ± 0.25 | 2.43 ± 0.15 | 2.27 ± 0.15 | 1.93 ± 0.25 | 1.6 ± 0.21 | 2.53 ± 0.25 | 2.03 ± 0.21 |
| | Supporting cell (μm) | 2.7 ± 0.2 | 2.7 ± 0.36 | 2.73 ± 0.12 | 2.4 ± 0.26 | 2.77 ± 0.25 | 0.67 ± 0.15 | 2.63 ± 0.15 | 2.73 ± 0.25 | 2.97 ± 0.15 | 2.57 ± 0.15 |
| Coconut odor | | Absent | Absent | Absent | Absent | Absent | Absent | Absent | Absent | Absent | Absent |
| Growth characteristics on PDA | Growth at 30 °C 72 h | MG | SG | SG | SG | SG | SG | SG | MG | SG | MG |
| | Growth at 40 °C 72 h | Absent | Present | Absent | Absent | Absent | Absent | Absent | Absent | Absent | Absent |
| | Conidia first observed (h) at 30 °C | 40 h | 28 h | 43 h | 40 h | 42 h | 48 h | 50 h | 42 h | 42 h | 42 h |
| | Sporulation on PDA 40 °C 72 h | Absent | Absent | Absent | Absent | Absent | Absent | Absent | Absent | Absent | Absent |
| | Colony reverse color | Cream | Yellow | Cream | Yellow | Cream | Yellow | Cream | Cream | Cream | Cream |
| | Colony radius at 30 °C 24 h (cm) | 2.77 ± 0.06 | 3.07 ± 0.06 | 3.03 ± 0.06 | 3.37 ± 0.06 | 2.3 ± 0.06 | 2.57 ± 0.06 | 2.13 ± 0.06 | 1.87 ± 0.15 | 2.3 | 2.67 ± 0.06 |
| | Colony radius at 30 °C 48 h (cm) | 4.23 ± 0.06 | 5.07 ± 0.12 | 4.8 ± 0.1 | 5.57 ± 0.06 | 4.8 | 5.17 ± 0.12 | 5.27 ± 0.06 | 4.23 ± 0.06 | 5.13 ± 0.06 | 4.23 ± 0.06 |
| | Growth at pH-3 30 °C 72 h (cm) | SG | MG | SG | SG | SG | SG | SG | WG | SG | SG |
| | Growth at pH-7 30 °C 72 h (cm) | SG | SG | SG | SG | SG | SG | SG | SG | SG | SG |
| | Growth at pH-9 30 °C 72 h (cm) | MG | SG | SG | SG | SG | SG | SG | MG | SG | SG |
| | *Trichoderma* radial growth rate (μm $h^{-1}$) | 882 | 1056 | 1000 | 1159 | 1000 | 1076 | 1097 | 882 | 1069 | 882 |
| | *Trichoderma* radial growth rate vs *S. rolfsii* (μm $h^{-1}$) | 325 | 289 | 325 | 356 | 558 | 689 | 597 | 564 | 558 | 547 |
| % inhibition of *S. rolfsii* | | 43.33 | 38.52 | 43.33 | 47.41 | 74.44 | 91.85 | 79.63 | 75.19 | 74.44 | 72.96 |

SG-Strong growth, MG moderate growth, WG weak growth.

[a] Data represented as means and their standard deviations from three replicates.

Geneious Pro 5.6. For identifying the species, bioinformatic tools available online from the International Subcommission on *Trichoderma* and *Hypocrea* (ISTH, www.isth.info), *Tricho*KEY v. 2.0.based on oligonucleotide barcode within the ITS1 and ITS2 sequences, *Tricho*MARK to analyze both ITS and *tef*1 sequences, *Tricho*BLAST to detect sequence similarity of the vouchered sequences in the ITS region and the largest 4th intron sequence of *tef*1 gene and Blastn from National Centre for Biotechnology information (NCBI) were used.

### 2.4. Genomic fingerprinting by RAPD and AFLP

A set of 20 RAPD primers (OPG series, Operon Technologies, USA) were tested across the 10 selected isolates of *Trichoderma*. All PCR reagents were procured from Merck Genei, Bangalore, India. RAPD assay was performed according to Sharma et al. (2009). The amplified products were resolved on a 2% agarose gel containing 0.5 μg $ml^{-1}$ ethidium bromide and photographed with the gel documentation system (Alpha Imager, Alpha Innotech, San Leandro, CA, USA).

AFLP assay was performed using the protocol described by Nath et al. (2012). Five pre-screened selective primer pairs *viz*., E+AT/T+AA, E+GA/T+GT, E+AG/T+AT, E+AT/T+AC, E+TG/T+TC were used for the amplification. At least two replicates of the amplification assay (RAPD and AFLP) were performed with template DNA from two different DNA extractions to ensure the consistency of each band.

All clearly detectable RAPD bands were scored for their presence (1) or absence (0). In order to ensure credibility only reproducible and well defined bands were scored. The RAPD and AFLP fingerprint data were introduced in the form of a binary matrix and a dendrogram of genetic relatedness among the isolates was generated as described under Section 2.2.

### 2.5. In vitro assessment of antagonistic activity

The antagonistic potential of the *Trichoderma* isolates against the plant pathogenic fungus, *S. rolfsii* were evaluated *in vitro* using three different methods.

#### 2.5.1. Direct confrontation assay

Discs of 5 mm were cut from the growing margin of each culture were used to inoculate PDA plates and incubated in dark at room temperature for all the *in vitro* tests. The inhibitory action of the isolates were first evaluated by the direct confrontation assay where each plate received two discs, one from the pathogen and one from each *Trichoderma* isolate, placed at a distance of 6 cm from each other. The antagonists were classified based on Bell et al. (1982) scale of antagonism classes.

#### 2.5.2. Effect of diffusible metabolites

The second test was performed to determine the inhibitory role of the diffusible metabolites produced by *Trichoderma* isolates on *S. rolfsii*. *Trichoderma* discs were inoculated on the center of PDA plates covered with sterile cellophane membrane and incubated for 5 days. The cellophane membranes were then aseptically removed and *S. rolfsii* disc was inoculated and cultured on the same plate (Dennis and Webster, 1971a). Plates without the prior inoculation of *Trichoderma* under the same conditions described above served as the control.

#### 2.5.3. Effect of volatile metabolites

The third method was designed to measure the role of the volatile metabolites produced by the *Trichoderma* isolates against the pathogen. The PDA plates were inoculated centrally by each *Trichoderma* isolate and the lid of the plate was replaced by the bottom dish of another PDA plate inoculated centrally with the pathogen disc. The two plates were sealed using an adhesive tape (Dennis and Webster, 1971b).The growth of *S. rolfsii* was recorded after 24 h, 48 h, and 72 h.

The percentage inhibition of the pathogen compared to control was calculated for both inhibitory diffusible and volatile metabolite assay. The individual experiment was repeated thrice and the results were compared by ANOVA using Fisher's LSD tests.

### 2.6. Enzyme induction and activity assays

The extracellular lytic enzyme production was induced by cultivating each *Trichoderma* isolate in Czapek's broth amended with 1% (w/v) *S. rolfsii* mycelium as the carbon source. The conidia suspension ($10^6$ conidia $ml^{-1}$) was used to inoculate 250 ml Erlenmeyer flasks containing 75 ml medium and was incubated at 150 rpm for 120 h at 28 °C. The biomass was then harvested by filtration and centrifugation at 13,307 g at 4 °C for 15 min. The supernatant obtained was used directly for the enzyme assay. The key lytic enzymes, N-acetyl hexosaminidase (exochitinase) and β 1,3 glucanase activity assays were done as described by Larralde-Corona et al. (2008).

The crude extract obtained was concentrated by 80% ammonium sulfate precipitation and was dialyzed against a 50 mM acetate buffer (pH 5.5). The protein profile of the crude extracts so obtained was assessed with sodium dodecyl sulfate–poly acrylamide gel electrophoresis (SDS–PAGE), according to the method described by Laemmli (1970).

### 2.7. Evaluation of the antagonistic efficacy of screened isolates using in vivo tests

The screened *Trichoderma* isolates, Tr9 and Tr10, selected based on the *in vitro* results were tested *in vivo* to study their efficacy in controlling collar rot of *Amorphophallus* caused by *S. rolfsii*. A pot experiment was laid out in completely randomized design with three treatments replicated five times such that 6 plants were used per replica. The treatments fixed were soil and tuber application of Tr9 plus pathogen challenge (T1), soil and tuber application of Tr10 plus pathogen challenge (T2) and control with pathogen challenge alone (T3). For soil application, *Trichoderma* enriched cow dung ($10^8$ cfu $g^{-1}$) was applied to each pot as a part of potting mixture and the tuber treatment (250 g) was done by dipping them for 2 h in cow dung slurry containing *Trichoderma* ($10^8$ cfu $ml^{-1}$). Cow dung without *Trichoderma* was used for the control treatments. The tuber pieces were then planted in pots containing the potting mixture of soil, sand and cow dung mixed in the ratio 2:1:1. A 2 × 2 cm of *Amorphophallus* tuber piece inoculated with *S. rolfsii* for 7 days at 28 °C in a humid chamber was applied to pots 5 months after planting for pathogen attack. Number of plants collapsed due to collar rot was counted on alternate days for 2 weeks and the mortality rate was calculated. The percentage of inhibition of the disease by the *Trichoderma* isolates was calculated by subtracting mortality rate of the treatments from those of the respective controls, dividing that by mortality rate of the controls and multiplying by 100. The results were analyzed by ANOVA and Duncan's test.

## 3. Results

### 3.1. Morphological characterization

All the 10 isolates were successfully identified using the morphological key program (http://nt.ars-grin.gov/taxadescriptions/keys/*Trichoderma*Index.cfm). The isolates used in the study

**Table 2**
Identification details of *Trichoderma* spp. used in this study.

| Isolates | Closest relative and their accession number in public databases | | | | Gen bank accession number | Molecular identification | Morphological identity |
|---|---|---|---|---|---|---|---|
| | NCBI | % identity | ISTH | % identity | | | |
| Tr4[a] | *T. asperellum* JQ617295.1 | 99 | *T. asperellum* CBS433.97 | 98 | KC859425 | *T. asperellum* | *T. asperellum* |
| Tr4[b] | *T. asperellum* JF964995.1 | 99 | *T. asperellum* CBS433.97 | 91 | KC572115 | | |
| Tr5[a] | *T. longibrachiatum* KC009811.1 | 100 | *T. longibrachiatum* ATCC18648 | 100 | KC859426 | *T. longibrachiatum* | *T. longibrachiatum* |
| Tr5[b] | *T. longibrachiatum* EU280046.1 | 99 | *T. longibrachiatum* GJS88-81 | 98 | KC572116 | | |
| Tr6[a] | *T. asperellum* KC569358.1 | 100 | *T. asperellum* CBS433.97 | 99 | KC859427 | *T. asperellum* | *T. asperellum* |
| Tr6[b] | *T. asperellum* JF964995.1 | 99 | *T. asperellum* CBS433.97 | 91 | KC572117 | | |
| Tr7[a] | *H. lixii* AB563725.1 | 99 | *T. harzianum* DAOM231412 | 99 | KC859428 | *T. harzianum* | *T. harzianum* |
| Tr7[b] | *H. lixii* EF191321.1 | 99 | *T. harzianum* GJS92-61 | 90 | KC572118 | | |
| Tr8[a] | *T. asperellum* KC561081.1 | 96 | *T. asperellum* CBS433.97 | 98 | KC859429 | *T. asperellum* | *T. asperellum* |
| Tr8[b] | *T. asperellum* JF964995.1 | 99 | *T. asperellum* CBS433.97 | 89 | KC572119 | | |
| Tr9[a] | *H. lixii* JQ411364.1 | 99 | *T. harzianum* CPK839 | 99 | KC859430 | *T. harzianum* | *T. harzianum* |
| Tr9[b] | *T. harzianum* AF443945.1 | 98 | *T. harzianum* GJS00-21 | 99 | KC572120 | | |
| Tr10[a] | *T. asperellum* KC898194.1 | 99 | *T. asperellum* CBS433.97 | 99 | KC859431 | *T. asperellum* | *T. asperellum* |
| Tr10[b] | *T. asperellum* EU077228.1 | 99 | *T. asperellum* CBS433.97 | 100 | KC572121 | | |
| Tr11[a] | *T. asperellum* JX677935.1 | 98 | *T. asperellum* CBS433.97 | 99 | KC859432 | *T. asperellum* | *T. asperellum* |
| Tr11[b] | *T. asperellum* JF964995.1 | 98 | *T. asperellum* CBS433.97 | 89 | KC572122 | | |
| Tr14[a] | *T. asperellum* JX677934.1 | 99 | *T. asperellum* CBS433.97 | 99 | KC859433 | *T. asperellum* | *T. asperellum* |
| Tr14[b] | *T. asperellum* JF964995.1 | 98 | *T. asperellum* CBS433.97 | 91 | KC5721123 | | |
| Tr15[a] | *T. asperellum* JX677935.1 | 99 | *T. asperellum* CBS433.97 | 99 | KC859434 | *T. asperellum* | *T. asperellum* |
| Tr15[b] | *T. asperellum* JF964995.1 | 99 | *T. asperellum* CBS433.97 | 89 | KC572124 | | |

[a] ITS.
[b] *tef*1.

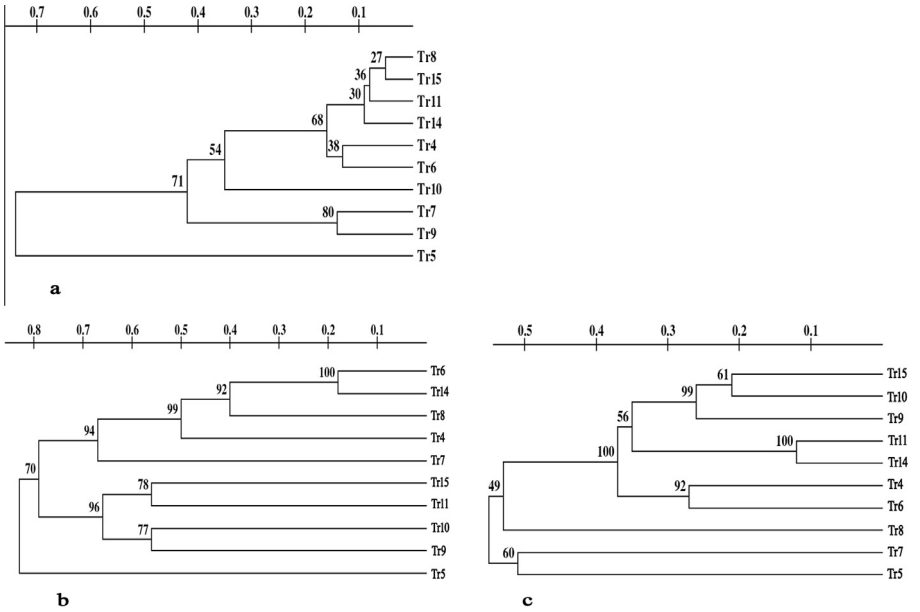


**Fig. 1.** UPGMA dendrogram of (a) Phenotype (b) RAPD (c) AFLP.

corresponded to one of the following species: *Trichoderma asperellum* (7 isolates), *Trichoderma harzianum* (2 isolates) and *Trichoderma longibrachiatum* (1 isolate) (Table 2). The quantitative and qualitative phenotypic data (Table 1) was transformed to build an unweighted pair group method with arithmetic mean (UPGMA) based dendrogram (Fig. 1a). The dendrogram divided the isolates into two clades; Clade I contained only one isolate, Tr5, the sole *Trichoderma* which was able to grow at 40 °C for 3 days. Clade II includes two sub clades, one represented the *T. asperellum* group while the other contained species of *T. harzianum.*

### 3.2. Molecular identification

The morphological identification was complemented by molecular identification based on ITS and *tef*1 sequence analyses. A single band in the range of 600 bp and 300 bp was amplified from each isolate for ITS and *tef*1 regions respectively. The study revealed the identification of three representative species as in the morphological identification. In general, the isolates on BLAST analyses showed 89–100% identity to the available sequences in public databases *viz.*, ISTH and NCBI. The Gen bank accession

**Table 3**
*In vitro* antagonistic activity of *Trichoderma* isolates against *S. rolfsii* based on Bell's scale; percent inhibition of growth of pathogen when grown on metabolites of *Trichoderma* isolate; exochitinase and β 1,3 glucanase activity per ml of culture filtrate of each isolate when grown in medium containing *S. rolfsii* mycelium as the sole C source.

| Isolates | Percentage of inhibition of *S. rolfsii* by diffusible metabolites | Exochitinase (μmol $min^{-1}$ $ml^{-1}$) | β 1,3 glucanase (nmol $min^{-1}$ $ml^{-1}$) | Bell's scale of classification |
|---|---|---|---|---|
| Tr4 | $42.71^{f}$ | $0.02^{h}$ | $3.98^{e}$ | Class III |
| Tr5 | $33.46^{h}$ | $0.02^{h}$ | $3.81^{e}$ | Class V |
| Tr6 | $40.38^{g}$ | $0.05^{f}$ | $6.46^{c}$ | Class III |
| Tr7 | $19.82^{i}$ | $0.037^{g}$ | $3.81^{e}$ | Class V |
| Tr8 | $57.25^{e}$ | $0.08^{de}$ | $6.13^{cd}$ | Class II |
| Tr9 | $98.51^{b}$ | $0.14^{a}$ | $5.47^{d}$ | Class I |
| Tr10 | $73.38^{d}$ | $0.12^{b}$ | $6.46^{c}$ | Class II |
| Tr11 | $86.45^{c}$ | $0.08^{d}$ | $7.29^{b}$ | Class II |
| Tr14 | $99.96^{a}$ | $0.09^{c}$ | $5.97^{cd}$ | Class II |
| Tr15 | $100^{a}$ | $0.08^{e}$ | $8.45^{a}$ | Class II |

Values in the same column followed by different letters are significantly different (Fisher's LSD, $p < 0.05$).
Classes of antagonism (based on Bell et al., 1982): Class I: *Trichoderma* grew and overlap the colony of pathogen and the whole surface of media; Class II: *Trichoderma* grew and it covered 2 of 3 of the surface of media; Class III: *Trichoderma* and pathogen colonized each half of the plate and did not dominate each other; Class IV: the pathogen grew and covered 2 of 3 of the surface of media; Class V: the pathogen overgrows completely on the surface of media.

number of all the isolates for the ITS and *tef*1 sequences and their corresponding identities to the available sequences are summarized in Table 2.

### 3.3. RAPD and AFLP analysis

A total of 229 bands were produced with an average frequency of 22.9 bands per primer. OPG16 yielded the maximum number of bands while minimum number was given by OPG3. All the bands obtained were polymorphic revealing a 100% polymorphism (Figs. A1 and A2). The dendrogram constructed to estimate the genetic relationship between the isolates is presented in Fig. 1b. The dendrogram had a profile similar to phenotypically based dendrogram with Tr5 forming a single clade and the rest of the isolates constituted the other clade. The isolates Tr9, Tr10, Tr11 and Tr15 which presented stronger antagonistic activity represented one subclade of Clade II while the weaker isolate Tr4, Tr5, Tr6 and Tr7 formed the other subclade with the exception of Tr8, one of the stronger antagonist was also included in this group.

Using the five primer combinations, a total of 303 bands were observed on AFLP analysis, of which 290 were polymorphic. The polymorphic bands varied from 40 to 51 with an average of 48.33 per primer (Fig. A3). The percent polymorphism ranged from 89.09% to 100%. The UPGMA dendrogram had two major clades, Clade I consisted of predominantly *T. asperellum* species with the exception of Tr9 (*T. harzianum*) and Clade II contained two species, *T. longibrachiatum* (Tr5) and *T. harzianum* (Tr7) (Fig. 1c). The AFLP profile could associate the isolates based on their antagonistic potential with the best isolates forming a single clade and less potent isolates constituted the other sub clade of Clade II. However, the dendrogram failed to group Tr8 among the potent isolates.

### 3.4. In vitro antagonistic activity

The isolate Tr9 overgrew the colony of *S. rolfsii* and completely covered the plate while the isolates Tr8, Tr10, Tr11, Tr14, and Tr15 grew two thirds of the surface of the medium and inhibited the pathogen. Tr4 and Tr6 occupied only half of the plate and had no dominance on *S. rolfsii* and vice versa. The pathogen overgrow completely on Tr5 and Tr7 covering the total area of culture plate. The classification of the isolates in relation to antagonism and overlapping of the colony is presented in Table 3.

On testing the strains for their ability to produce diffusible metabolites against *S. rolfsii*, Tr8, Tr9, Tr10, Tr11, Tr14, and Tr15 inhibited the pathogen by more than 50%, with the 100% inhibition shown by Tr15. Moderate inhibition of about 25–50% was shown by the isolates Tr4, Tr5, and Tr6 while least activity was measured for Tr7 (19.82%) (Table 3). The third test to determine the role of volatile metabolites showed no inhibitory activity as the pathogen grew profoundly as that of the control plate.

### 3.5. Biochemical analyses

The ten isolates of *Trichoderma* tested for antagonism against *S. rolfsii* produced and secreted exochitinase and β 1,3 glucanase, on induction. The highest production of exochitinase was observed with Tr9 while Tr4 and Tr5 showed the lowest activity. All the isolates produced considerably low amount of β 1,3 glucanase, Tr4, Tr5 and Tr7 with the minimum activity and Tr15 produced the maximum (Table 3). As a general trend, a positive correlation was obtained with the degree of antagonism to *S. rolfsii* and the lytic enzymes produced by the *Trichoderma* isolates against the mycelium of the pathogen when given as the sole carbon source.

A specific protein profile was observed when the precipitated dialyzed crude protein extract from the induced cultures of all the *Trichoderma* isolates (Fig. A4). An attempt was made to compare the molecular weights of each band to the already published molecular weights of enzymes of *Trichoderma* with biocontrol activity (Larralde-Corona et al., 2008). Molecular weights of 10 bands could be associated to previously identified proteins of *Trichoderma* (Table 4). The banding pattern among the isolates also varied noticeably. Tr9, the most isolate which showed maximum antagonistic activity had the most complex protein profile.

### 3.6. Pot experiment

The soil and tuber application of both *Trichoderma* strains (Tr9 and Tr10) significantly reduced the collar rot incidence under artificial infection with *S. rolfsii*. The mortality rate shown Tr9 and Tr10 were 13.33% and 30% respectively. Tr9 reduced the disease incidence by 85.33% while Tr10 gave 66% reduction of collar rot incidence over control (Fig. 2). From this data, it is clear that Tr9 has a good potentiality of controlling the collar rot disease of *Amorphophallus* caused by *S. rolfsii*.

The most promising isolate, *T. harzianum* (Tr9) was deposited in National Collection of Industrial Microorganisms (NCIM) under the accession number NCIM 1373. Sequences corresponding to ITS1-5.8s-ITS2 rDNA and *tef*1 were deposited and available in the Gen bank database under the accession number given in Table 2.

**Table 4**
Representative protein profile of the isolates of *Trichoderma* grown in presence of *S. rolfsii* mycelium as sole C source.

| Molecular weight of proteins (kDa) | Isolates | | | | | | | | | | Possible protein | References |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Tr4 | Tr5 | Tr6 | Tr7 | Tr8 | Tr9 | Tr10 | Tr11 | Tr14 | Tr15 | | |
| 105 | – | – | – | – | – | × | – | – | – | – | N-acetyl-β-D glucosmainidase 102 kDa | Haran et al. (1995)[a] |
| 79 | – | – | – | – | – | × | × | – | – | – | β 1,3 endoglucanase 78 kDa | de la Cruz et al. (1995)[a] |
| 72 | – | – | – | – | × | – | – | – | × | – | N-acetyl-β-D glucosmainidase 73 kDa<br>β 1,3 exoglucanase 75 kDa<br>β 1,6 endoglucanase 74 kDa | Haran et al. (1995)[a]<br>Ramot et al. (2000)[a]<br>El-Katany et al. (2001)[b] |
| 63 | × | – | × | × | × | × | × | – | – | × | N-acetyl-β-D glucosmainidase 64 kDa | Ulhoa and Peberdy (1991)[b] |
| 43 | – | – | – | – | × | × | × | × | × | × | Endochitinase 42 kDa<br>chitobiosidase 40 kDa<br>β 1,6 endoglucanase 43 kDa | Carsolio et al. (1994)[a], Garcia et al. (1994)[a], Lorito et al. (1998)[a], de la Cruz et al. (1992)[b]<br>Harman et al. (1993)[a]<br>Lorito et al. (1995)[a] |
| 36 | – | – | – | – | – | × | × | × | – | – | Endochinase 37, 36 kDa | de la Cruz et al. (1992)[a], Viterbo et al. (2001)[a], de Marco et al. (2000)[a] |
| 34 | – | × | – | × | – | × | – | – | – | – | Endochitinase 33 kDa | Limon et al. (1995)[b], de la Cruz et al. (1992)[a], Haran et al. (1996)[a] |
| 30 | – | – | × | – | – | × | × | × | × | – | Alkaline protease 31 kdDa<br>Endochitinase 30, 31 kDa | Flores et al. (1995)[a]<br>Haran et al. (1996)[b] |
| 27 | – | × | – | × | – | – | – | × | – | × | Exochitinase 28 kDa | Deane et al. (1998)[a], Lorito et al. (1994)[b] |
| 24 | × | – | × | – | – | × | – | – | × | × | Chitinase 24 kDa | Krishnaveni et al. (1999)[b] |

[a] As reviewed by Viterbo et al. (2002).
[b] As reviewed by Markovich and Kononova (2003).

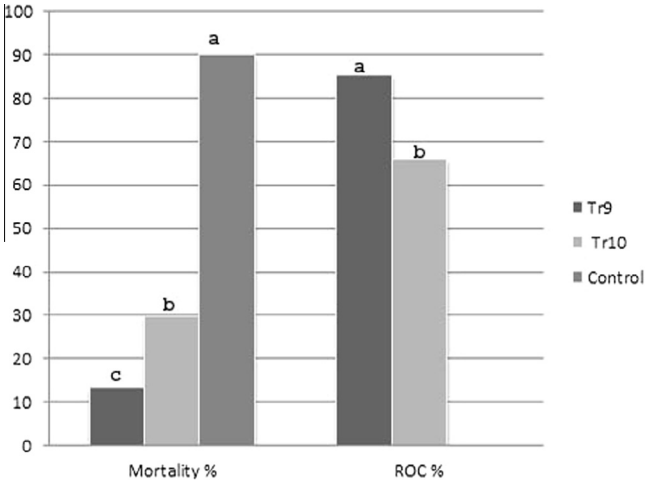


**Fig. 2.** *In vivo* evaluation of the antagonistic activity of Tr9 and Tr10 against collar rot infection of *Amorphophallus* by *Sclerotium rolfsii*. The data are presented as mortality percent and percentage of reduction of disease as compared to controls (ROC) without *Trichoderma*. ANOVA and Duncan's tests were performed separately. Bars designated by different small letters for each parameter are significantly different (Duncan, $p < 0.05$).

## 4. Discussion

Previous studies by Grondona et al. (1997), Hermosa et al. (2000) and Kullnig-Gradinger et al. (2002), attempted to combine the morphological and physiological data. However, only few reports are available on the use of combined datasets from morphological and molecular analyses (Lieckfeldt et al., 1998; Buhariwalla et al., 2005). Gajera and Vakharia (2010) and Sharma et al. (2009) presented characterization of *Trichoderma* that discussed the correlation of morphological, biochemical and molecular features of different isolates. A polyphasic approach to characterize *Trichoderma* against *Macrophomina phaseolina* was initiated by Larralde-Corona et al. (2008) which could clearly group isolates at their species level or antagonistic activity. Hence, in our study we also employed a similar strategy of characterization of *Trichoderma* against *S. rolfsii* that included the use of RAPD and AFLP analysis to support the molecular approach along with phenotypical and biochemical parameters. This is the first report of intensively characterizing *Trichoderma* strains against *S. rolfsii*, in an attempt to explore their biocontrol potential in managing collar rot disease.

The classical way for the identification of *Trichoderma* includes both morphological and molecular approaches (Lieckfeldt et al., 1998; Monteiro and Ulhoa, 2006). The morphological identification has become more convenient by the use of the online interactive key which employs both microscopic and macroscopic features of each isolate. This has proved to be a potential method for taxonomically identifying *Trichoderma* spp. although they require regular updates for the rapidly increasing number of species in the genus (Anees et al., 2010). As suggested by Samuels et al. (2006), a single gene is not sufficient to characterize species of *Trichoderma*, a multigene approach with two unlinked loci, a combination of ITS rDNA and *tef*1 sequences were employed for the identification of species in this study. The Gen bank database which is generally used however may contain some invalid entries (Druzhinina and Kubicek, 2005) and therefore could not be safely used. Therefore, the specific database for *Trichoderma* (ISTH) which contain the vouchered sequences were also included for sequence identification. A similar approach was employed in the previous studies also (Zhang et al., 2005; Migheli et al., 2009; Anees et al., 2010). Our results were consistent with the morphological

identification although some authors have discussed the lack of relationship between morphological and molecular (ITS) characterizations (Kullnig-Gradinger et al., 2002; Druzhinina and Kubicek, 2005).

The usefulness and reliability of direct confrontation assay in identifying the biocontrol potential of *Trichoderma* strains has been well recognized (Hermosa et al., 2000). Sanz et al. (2004) demonstrated semi-specificity in the interaction of *Trichoderma* with its host, as *Trichoderma* isolates belonging to the same species showed differential antagonistic activity when comparing the *in vitro* antifungal activity of several *Trichoderma* collection strains against spores of *Botrytis cinerea*. We also observed the differential inhibitory action among the isolates of *T. asperellum* and *T. harzianum* against *S. rolfsii*, the collar rot pathogen of *Amorphophallus*. Anees et al. (2010) also established in his study that the antagonism is not a property of species, as different strains of same species can exhibit varying potentials of biocontrol. So adding a functional line to characterize the mode of action of *Trichoderma* could render the inter and intra specific diversity of the antagonists (Migheli et al., 2009; Anees et al., 2010). The present study tried to characterize the mechanism of antagonism by dual culture assay and testing the strains for the production of water soluble and volatile metabolites that could inhibit *S. rolfsii*. The isolates that inhibited *S. rolfsii* in direct confrontation also produced diffusible inhibitory metabolites (Table 3) but none of them shown inhibition by volatile metabolites. This could suggest the role of antibiosis or production of catabolic enzymes as one of the major mechanism of antagonism that have already been reported (Harman et al., 2004).

Chitin and β-1,3-glucan are the main structural components of most fungal cell walls (Lorito et al., 1994). Studies by Bloomfield and Alexander (1967) observed that the acid hydrolysate of the hyphal walls of *S. rolfsii* consisted of glucose and glucosamine; these accounted for 18.3% and 61.0% of the weight of the walls, respectively, prepared from 72-h cultures indicating the presence of chitin and β-1,3-glucan in the cell wall. In our case, an elevated production of β-N-acetylhexosaminidase (exochitinase) was observed compared to β-1,3-glucanase when *S. rolfsii* mycelium was given as the sole carbon source, indicating the presence of high amount of chitin in the hyphae of *S. rolfsii* (Table 3).

The cell wall degrading enzymes, such as exochitinases and β-1,3-glucanases, has been known to play a major role in mycoparasitism (Kubicek et al., 2001).The present study revealed a positive relationship between the enzymatic activities of β-1,3-glucanase and exochitinase with the degree of antagonism measured in the confrontation assay. Similar results of positive association of extracellular enzymatic activity and degree of inhibition were obtained by Larralde-Corona et al. (2008). A stronger positive correlation ($r_s = 0.957$) was observed in our study for the exochitinase when compared to β-1, 3-glucanase activity ($r_s = 0.434$). *T. harzianum* (Tr9), the strain with the highest antagonistic activity also had the greatest exochitinase activity (0.14 μmol $min^{-1}$ $ml^{-1}$) while *T. longibrachiatum* (Tr5), which had the lowest inhibitory role also produced the low amount of exochitinase (0.02 μmol $min^{-1}$ $ml^{-1}$) (Table 3). This hold up the fact that the lytic enzymes produced by *Trichoderma* species play an important role in the destruction of plant pathogens, as observed earlier by Haran et al. (1996).

Each isolate showed distinct protein profiles with molecular weights similar to those of the *Trichoderma* proteins involved in antagonistic activity. We could relate the bands observed with different N-acetyl-β-D glucosaminidases, endochitinases, chitobiosidases, β-1,3 exoglucanases, β-1,3 endoglucanase, β-1,6 endoglucanase and protease (Table 4). The isolates with good biocontrol performance excreted more protein as evidenced by the SDS–PAGE profile with Tr9 has the most complex profile (Fig. A4). The proteins of molecular weights 42 kDa and 37, 36 kDa, were specifically found in the best antagonistic isolates and is related to endochitinase (Table 4).

The combination of RAPD and AFLP markers was used to identify the isolates of *Trichoderma* used in this study. The highest growth inhibition of the pathogen shown by Tr9 and Tr10 represented same cluster for both RAPD and AFLP dendrogram instead of being different species (Fig. 1). These isolates also had the greatest exochitinase activity and complex protein profile. Though, Tr7 and Tr9 belong to the same species are grouped under different cluster showing the interspecific and intraspecific genetic variation among the isolates. Studies by Goes et al. (2002), Larralde-Corona et al. (2008) and Gajera and Vakharia (2010) supports our findings. The morphological dendrogram (Fig. 1) revealed grouping of isolates at species level while the RAPD and AFLP profiles lead mostly the grouping based on antagonism with the exception of Tr8 and Tr14 for RAPD and Tr8 for AFLP. Grouping related to species level can also be estimated with these markers except for *T. harzianum* (Tr7 and Tr9).We also tested the efficacy of the screened isolates (Tr9 and Tr10) in controlling the collar rot incidence. Our results, based on the soil and tuber treatment of these isolates indicated the comparative success of Tr9 in disease incidence reduction of 85% (Fig. 2). Therefore, the overall results demonstrated an effective reduction of collar rot incidence of *Amorphophallus* caused by *S. rolfsii* under *in vitro* as well as pot experiment.

In conclusion, the present study allowed us to integrate and correlate the morphological, biochemical and molecular characteristics of different isolates of *Trichoderma* having differential ability in inhibiting *S. rolfsii*. This approach provides new insights for the selection of *Trichoderma* as biological control agents against fungal plant pathogens.

## Conflict of interest

All the authors of this manuscript states that there is no sources of conflict of interests to declare.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocontrol.2015.07.001.

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