Identification, characterization and phylogenetic analysis of antifungal *Trichoderma* from tomato rhizosphere

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**Abstract**

The use of *Trichoderma* isolates with efficient antagonistic activity represents a potentially effective and alternative disease management strategy to replace health hazardous chemical control. In this context, twenty isolates were obtained from tomato rhizosphere and evaluated by their antagonistic activity against four fungal pathogens (*Fusarium oxysporum* f. sp. *lycopersici*, *Alternaria alternata*, *Colletotrichum gloeosporoides* and *Rhizoctonia solani*). The production of extracellular cell wall degrading enzymes of tested isolates was also measured. All the isolates significantly reduced the mycelial growth of tested pathogens but the amount of growth reduction varied significantly as well. There was a positive correlation between the antagonistic capacity of *Trichoderma* isolates towards fungal pathogens and their lytic enzyme production. The *Trichoderma* isolates were initially sorted according to morphology and based on the translation elongation factor 1-α gene sequence similarity, the isolates were designated as *Trichoderma harzianum*, *T. koningii*, *T. asperellum*, *T. virens* and *T. viride*. PCA analysis explained 31.53, 61.95, 62.22 and 60.25% genetic variation among *Trichoderma* isolates based on RAPD, REP-, ERIC- and BOX element analysis, respectively. *ERG*-1 gene, encoding a squalene epoxidase has been used for the first time for diversity analysis of antagonistic *Trichoderma* from tomato rhizosphere. Phylogenetic analysis of *ERG*-1 gene sequences revealed close relatedness of *ERG*-1 sequences with earlier reported sequences of *Hypocrea lixii*, *T. arundinaceum* and *T. reesei*. However, *ERG*-1 gene also showed heterogeneity among some antagonistic isolates and indicated the possibility of occurrence of squalene epoxidase driven triterpene biosynthesis as an alternative biocontrol mechanism in *Trichoderma* species.

**Keywords:** Antagonism, Biocontrol, Diversity, Ergosterol, Tomato, *Trichoderma*

**Background**

The genus *Trichoderma* has gained immense importance in past several decades due to its antagonistic ability against wide range of plant pathogens and growth promotion in crop plants. Some species of *Trichoderma* viz., *Trichoderma harzianum*, *T. viride*, *T. virens* and *T. koningii* are well known antagonists and are being utilized to control plant pathogens under field conditions (Solanki et al. 2011; Srivastava et al. 2012; Galarza et al. 2015). Promising *Trichoderma* isolates have different mechanisms or combination of direct parasitism, competition for nutrients, stimulators of plant health, or inducers of plant systemic resistance against various pathogens (Harman et al. 2004; Anees et al. 2010; Woo et al. 2014; Jain et al. 2015; Rai et al. 2016). A plethora of antagonistic *Trichoderma* isolates have been identified by several researchers from different places around the world, having history of varied climate, soil type, cropping system, etc., which differ in their innocuousness and efficacy as biocontrol agents (Sharma et al. 2009; Blaszczyk et al. 2011; Martínez-Medina et al. 2014; Galarza et al. 2015; El_Komy et al. 2015). Therefore, the site specific recommendations are being made according to the fitness potential of a particular isolate for higher efficacy and effectiveness. Despite the commercial successes of these biocontrol agents, the major limitations remain their restricted efficacy and inconsistency under field...
conditions. Consequently, more efficient *Trichoderma* isolates with high antagonistic potential capabilities are needed for successful biological control systems.

Due to the ecological importance of *Trichoderma* spp. and their application as a biocontrol agent in the field, it is important to understand their biodiversity. However, accurate species identification based on morphology is difficult due to the paucity and similarity of morphological characters and increasing numbers of morphologically cryptic species (Kullnig et al. 2001). This has already resulted in incorrect identification. In recent years, the usefulness of molecular markers such as random amplified polymorphic DNA (RAPD) and repetitive-element polymerase chain reaction (REP-PCR) in resolving species differences among microbial species are also well documented (Sharma et al. 2009; Solanki et al. 2013; Srivastava et al. 2014; Singh et al. 2014; Kashyap et al. 2015). RAPD utilized PCR to amplify DNA segments with single primer of arbitrary nucleotide sequence generating fragments by hybridizing with compatible regions of DNA and amplifying the regions where the primers are in correct orientation and appropriately spaced (100–2500 bp). However, REP-PCR uses oligonucleotide primers complementary to repetitive sequences dispersed throughout the genome. Using PCR, this method amplifies diverse regions of DNA flanked by the conserved repetitive sequences, leading to ampiclon patterns specific for an individual bacterial and fungal strain. Three different families of repetitive sequences include: the 35–40 bp repetitive extragenic pallindromic (REP) sequence, the 124–127 bp enterobacterial repetitive intergenic consensus (ERIC) sequence and 154 bp BOX (composed of the box A, B and C subunits) element. These sequences appear to be located in distinct, intergenic positions around the genome elements (Mohapatra et al. 2007). Methods based on such repetitive elements have also been used for studying the diversity in the ecosystem, presenting the phylogenetic relationship between strains and discriminating between microorganisms those are genetically close to each other (Rai et al. 2015; Kashyap et al. 2016). Unfortunately, these methods have not been extensively used for the differentation of *Trichoderma* spp. Since, species of *Trichoderma* are reported as the causal agent of green mould disease (Ospina-Giraldo et al. 1998), the understanding of the nature and diversity of *Trichoderma* is critical for its widespread use against phytopathogenic fungi as there could be the risk of unwanted disease on non-target hosts. Under such situations, it is valuable to establish patterns of gene flow, as well as to develop a fingerprint of *Trichoderma* isolates. Diversity studies have recently been undertaken to assess its ecological specialization. Several studies reported about a series of new isolates as well as new phylogenetic species of *Trichoderma* in a series of natural ecosystems (Zachow et al. 2009; Kőrmöczi et al. 2013). On the other hand, only a few studies were focusing on agricultural environments. However, the results of these studies demonstrated that besides the natural ecosystems, the investigation of agricultural soils also reveals important information about *Trichoderma* biodiversity. The practical impact of such studies is that the rhizosphere of agricultural soils may be an ideal source of beneficial strains with biocontrol potential. Based on these studies, we speculate that the species composition, distribution, and genetic structure of *Trichoderma* on the tomato rhizosphere may be different. The confirmation of the differences will help in revealing the biodiversity, origin, and evolutionary processes of *Trichoderma* under different biological niches.

Recent evidences indicated the importance of the sterol biosynthetic pathway in inducing plant defense-related gene expression in both the antagonistic fungus and the plant (Cardoza et al. 2011; Malmierca et al. 2013; Cardoza et al. 2014). The structural and functional analysis of genes involved in the synthesis of ergosterol especially intermediates, such as squalene could provide additional strategies to improve the ability of biocontrol of the *Trichoderma* strains. To best of the knowledge, there are no reports available on the diversity analysis of ergosterol producing antagonist *Trichoderma* species using ERG1 gene, encoding a squalene epoxidase, a key enzyme in the biosynthesis of triterpene derivatives (e.g. ergosterol) from tomato rhizosphere. Thus, to test above mentioned hypothesis, attempts have been made to investigate the species distribution of *Trichoderma* associated with tomato plants. The comparison of the genetic structure between antagonist *Trichoderma* isolates was carried out by molecular (RAPD, REP, ERIC and BOX markers), and biochemical (production of cell wall degrading enzymes) markers. Sequencing based on the characterization of squalene epoxidase (*ERG1*) gene in antagonist isolates was performed to get preliminary clues about the role of squalene epoxidase driven triterpene biosynthesis in biocontrol mechanisms of tested isolates.

**Methods**

**Sampling and identification of *Trichoderma* isolates**

Twenty isolates of *Trichoderma* were obtained from healthy tomato (*Solanum lycopersicum* cv. VL tamatar 4) rhizosphere (Table 1). Ten healthy plants (~55 days post transplanting) with their roots and rhizospheric soil were randomly sampled and immediately transported to the laboratory. The soil particles attached to roots were carefully collected after uprooting plants, stored at 4 °C and processed within 24 h of collection. Root adhered
Table 1 Identification, origin, NCBI Genebank accession numbers, cell wall-degrading enzymes and antagonistic effect of *Trichoderma* isolates from tomato rhizosphere against fungal plant pathogens

| Code  | Isolate(s)                  | Region          | GenBank accession numbers | Cell wall-degrading enzymes | Mycelia inhibition over control (%) |
|-------|-----------------------------|-----------------|---------------------------|-----------------------------|--------------------------------------|
|       |                             |                 |                           | EF-1α                       | FOL                                 |
|       |                             |                 |                           | ERG1                        | AA                                  |
|       |                             |                 |                           | Chitinase*                  | CG                                  |
|       |                             |                 |                           | β-1,3 glucanase*             | RS                                  |
| UNT60 | *Trichoderma harzianum*     | U.S. Nagar, Uttarakhand | KF360991 KT89041         | *40.0 ± 1.4 ±          | 58.54 ± 2.41  |
|       |                             |                 |                           | +                         | 58.89 ± 2.14  | 59.80 ± 1.88  | 53.30 ± 1.99  |
| UNT64 | *T. harzianum*              | U.S. Nagar, Uttarakhand | KF360992 KT89042         | 69.00 ± 3.54 ±         | 62.45 ± 1.56  | 61.50 ± 1.91h | 64.50 ± 2.11cd | 62.78 ± 1.88bcd |
| UNT68 | *T. harzianum*              | U.S. Nagar, Uttarakhand | KF360993 KT89043         | 76.56 ± 3.95 ±         | 77.94 ± 2.21  | 79.47 ± 1.88a | 73.94 ± 1.88a | 69.23 ± 1.35a |
| NAT69 | *T. harzianum*              | Nanital, Uttarhankd | KF360994 KT89044         | 53.33 ± 1.44 ±         | 57.65 ± 1.12g | 53.98 ± 1.34g | 59.50 ± 1.77g | 54.80 ± 1.66g |
| NAT70 | *T. harzianum*              | Nanital, Uttarhankd | KF360995 KT89045         | 37.0 ± 1.20 ±          | 62.34 ± 1.21   | 62.50 ± 1.99ph | 60.89 ± 1.55g | 62.79 ± 1.64bcd |
| ALT73 | *T. harzianum*              | Almora, Uttarhankd | KF360996 KT89046         | 49.67 ± 2.01f          | 65.78 ± 1.54f  | 53.68 ± 1.86f | 64.70 ± 1.66cd | 63.65 ± 1.94f |
| DET89 | *T. harzianum*              | Dehradun, Uttarhankd | KF360997 KT89047         | 67.00 ± 3.93d          | 61.98 ± 1.34f  | 67.87 ± 1.54d | 62.56 ± 1.35g | 58.83 ± 1.51e |
| DET94 | *T. harzianum*              | Dehradun, Uttarhankd | KF360998 KT89048         | 69.00 ± 4.10d          | 74.35 ± 1.14d  | 65.50 ± 1.99d | 68.89 ± 2.01d | 67.89 ± 1.35d |
| HAT96 | *T. harzianum*              | Haridwar, Uttarhankd | KF360999 KT89049         | 73.60 ± 3.35  | 68.95 ± 1.21d  | 63.45 ± 1.35f | 60.54 ± 1.35b | 61.11 ± 1.91bcd |
| UNT38 | *T. koningii*               | U.S. Nagar, Uttarakhand | KF361001 KT89051         | 74.60 ± 3.55a         | 70.73 ± 1.12e  | 69.80 ± 1.36e | 68.90 ± 1.55b | 64.78 ± 1.35e |
| UNS63 | *T. koningii*               | U.S. Nagar, Uttarakhand | KF361002 KT89052         | 39.00 ± 1.99          | 52.98 ± 1.33  | 56.89 ± 1.25  | 56.90 ± 1.97  | 59.80 ± 2.11de |
| UNT13 | *T. asperellum*             | U.S. Nagar, Uttarakhand | KF361003 KT89053         | 56.56 ± 2.44d         | 55.80 ± 1.21f  | 61.89 ± 1.44f | 62.80 ± 1.25f | 59.80 ± 1.87de |
| UNT70 | *T. asperellum*             | U.S. Nagar, Uttarakhand | KF361004 KT89054         | 53.43 ± 1.66e         | 53.90 ± 1.44e  | 59.98 ± 0.99e | 60.70 ± 1.35f | 62.89 ± 1.76bcd |
| UNS28 | *T. virens*                 | U.S. Nagar, Uttarakhand | KF361005 KT89055         | 61.00 ± 1.32          | 64.30 ± 1.31e  | 67.50 ± 1.34d | 65.80 ± 1.97e | 60.80 ± 1.54de |
| UNS30 | *T. virens*                 | U.S. Nagar, Uttarakhand | KF361006 KT89056         | 73.67 ± 2.12c         | 70.89 ± 1.21c  | 75.60 ± 1.65c | 65.70 ± 1.54c | 64.58 ± 1.62c |
| NAS46 | *T. virens*                 | Nanital, Uttarhankd | KF361007 KT89057         | 44.00 ± 1.19g         | 65.80 ± 1.33c  | 60.80 ± 1.21c | 59.80 ± 1.35c | 57.50 ± 1.31f |
| ALS47 | *T. virens*                 | Almora, Uttarhankd | KF361008 KT89058         | 57.00 ± 2.37h         | 69.00 ± 1.54d  | 65.78 ± 1.35e | 61.90 ± 1.67e | 62.80 ± 1.15bc |
| UNT09 | *T. viride*                 | U.S. Nagar, Uttarakhand | KF361011 KT89061         | 59.90 ± 1.15          | 67.83 ± 1.34d  | 64.45 ± 1.44f | 60.85 ± 1.88f | 60.70 ± 1.25de |
| DET02 | *T. viride*                 | Dehradun, Uttarhankd | KF361012 KT89062         | 31.00 ± 1.25          | 72.05 ± 1.68c  | 68.89 ± 1.55cd | 64.44 ± 1.66de | 60.35 ± 1.35de |
| NAT03 | *T. viride*                 | Nanital, Uttarhankd | KF361013 KT89063         | 61.67 ± 2.03          | 61.80 ± 1.34c  | 64.50 ± 1.39f | 59.48 ± 1.95c | 60.45 ± 1.75de |

* Within columns, mean ± SE values with a common letter do not differ significantly (P < 0.05), according to DwMT test

* μmol of GlcNAc min⁻¹ mg⁻¹ protein

* nmol of glucose min⁻¹ mg⁻¹ protein
soil (10 g) was suspended in 90 ml of sterile distilled water and dilution plate technique was used for the isolation of *Trichoderma* spp. The suspensions from all samples were serially diluted (up to $10^{-5}$) and 100 μl of each dilution was added to sterile Petri dishes, in triplicates of each dilution, containing sterile Potato Dextrose Agar (PDA) medium. Streptomycin solution (1%) was added to the medium for preventing bacterial growth, before pouring into Petri plates. The plates were then incubated at 28 ± 1 °C. The isolates were characterized based on the monograph of Gams and Bissett (1998). For morphological analysis, isolates were grown on PDA at 28 ± 1 °C for 5–7 days. Radial growth was measured at 24 h intervals until colony covered the whole Petri dish. Growth rate was calculated as the 7 day average of mean daily growth (mm day$^{-1}$). All micro morphological data were examined from cultures grown on PDA for 5 days at 28 ± 1 °C. Microscopic observations were done using trinocular microscope (Axio Imager M2 microscope, Carl Zeiss, Germany). For examination of conidial morphology, cultures were washed with sterile water and drops of the suspension were placed on microscope slides and mixed with lactophenol/cotton blue to stain the conidia. Length and width were measured for 30 conidia per isolate. Conidial morphology and size were recorded after 7 days of incubation.

**Screening the antagonistic activity of *Trichoderma* isolates**

In vitro antagonistic potential of the biocontrol agent was evaluated against *Fusarium oxysporum* f. sp. lycopersici (FOL), *Alternaria alternata* (AA), *Colletotrichum gloeosporioides* (CG) and *Rhizoctonia solani* (AA) by Kumar et al. (2013a). Briefly, for each fungal isolates, fresh mycelium (~5 g) was dried on sterile blotter paper and was ground in liquid nitrogen to make a fine powder. This powder was taken in a centrifuge tube and 2× CTAB (hexadecyltrimethyl ammonium bromide) buffer (15 ml) was added in each tube separately. Extraction buffer contained (per 1 l) 2 g CTAB, 1 M Tris pH-8 (10 ml), 5 M NaCl (28 ml), 0.5 M EDTA (4 ml) with sterile distilled water (57 ml) and 1 ml β-mercaptoethanol. This was incubated in water bath at 65 °C for 30 min with intermittent shaking. The mixture was centrifuged at 13,000 rpm for 15 min at 4 °C to pellet the mycelium. Supernatant was taken into another Oakridge tube and an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added with slow inversion. The mixture was again centrifuged at 13,000 rpm for 15 min at 4 °C. The aqueous supernatant was taken in a fresh tube and added 0.6 volume isopropanol and was incubated at −20 °C overnight. After incubation, it was again centrifuged at 13,000 rpm for 20 min at 4 °C temperature. The supernatant was discarded and pellet was washed with 70% ethanol. The pellet was dissolved in 500 μl of TE buffer for the use in PCR and stored at −20 °C.

For the molecular identification of ergosterol producing isolates, *ERG1F* (5ʹ-CGCTCCGTCTTCTTC TTC TC-3ʹ) and *ERG1R* (5ʹ-CTCTTCTTCTCCGGTCTCC-3ʹ) primers were used. The PCR reaction was carried out in a 25-μl reaction mixture containing the following: 10× PCR buffer, 50 ng DNA template, 2 mM MgCl$_2$, 0.25 mM dNTP mixture and 0.25 μM each of primer, and one unit of *Taq* Polymerase (Bangalore Genie, India). Amplifications were performed in Thermal Cycler (G Storm GS4, Somerset, UK) under the following conditions: initial denaturation 5 min at 94 °C, 35 cycles of 45 s at 94 °C, 45 s at 58 °C, 1 min at 72 °C, with the final extension of 10 min at 72 °C.

Polymerase chain reaction (PCR) assay for translation elongation factor (*TEF-1a*) gene was conducted using primers TEF1-728 F and TEF1-986R (Al-Sadi et al. 2015). The PCR reactions were carried out in 25 μl reaction mixture containing 10× PCR buffer, 50 ng DNA template, 2 mM MgCl$_2$, 0.25 mM dNTP mixture and 0.25 μM each of primer, and one unit of *Taq* Polymerase (Bangalore Genie, India). Thermocycling was run with the following settings: heating at 94 °C (5 min); then 35 cycles of 94 °C (30 s), 60 °C (30 s) and 72 °C (90 s). The final extension was done at 72 °C for 10 min.

Molecular characterization of *Trichoderma* isolates was assessed by rep-PCR using the BOX1R, Rep1R-L, Rep21, ERIC-1R and ERIC-2F primers (Srivastava et al. 2014). All the PCR reactions were carried out in 25 μl reaction mixture containing 5× Gitschier buffer, 50 ng DNA template, 2 mM MgCl$_2$, 0.25 mM dNTP mixture and 0.25 μM each of primer, and one unit of *Taq* Polymerase (Bangalore Genie, India). Thermal Cycler (G Storm GS4, Somerset, UK) was programmed as an initial denaturation at
94 °C for 5 min, 40 cycles of 94 °C for 1 min, 36 °C for 1 min and 72 °C for 2 min and a final extension at 72 °C for 10 min.

For RAPD assay, the DNA extracted from tested isolates was amplified with the RAPD primers using the five RAPD primer set (Bangalore Genei, India). The thermal profile used was initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation step (94 °C for 1 min), annealing (47 °C, 1 min), extension (72 °C for 1.5 min), and a final extension step (72 °C for 7 min).

Amplified products were resolved in 2.0% agarose gels using 1× TAE buffer on a gel electrophoresis apparatus. Ethidium bromide (0.25 mg ml⁻¹) was used as an intercalating agent. The gel was run at 2 V cm⁻¹ of the length of gel till the bands resolved. The amplified bands, after separation on the gel, were visualized and documented using a gel documentation imaging system (Bio-Rad, USA).

**Statistical analysis**

Experimental data for conidial morphology and growth rate were analyzed using Duncan's multiple range test (DMRT). Standard errors were calculated for all mean values. All RAPD, ERIC, REP and BOX-PCR reactions were repeated to ensure validity of results. The presence or absence of individual, distinct, and reproducible bands was scored as '1' for presence and '0' for absence. Principle component analysis (PCA) was performed using XLSTAT software.

**Results**

**Antagonistic activity of Trichoderma isolates**

Antagonistic capabilities of the *Trichoderma* isolates were assessed by the growth inhibition of four fungal pathogens (FOL, AA, CG and RS) through the dual culture assay. In general, all the antagonistic isolates grew faster than pathogen. The interaction of biological control agents versus four different fungal pathogens showed significant differences in growth inhibition of the pathogen isolates (Table 1). Isolate UNT68 showed highest inhibition effect on the percent mycelia growth of FOL (77.94%), AA (79.47%), CG (73.94%) and RS (69.23%). Contrarily, isolate UNT70, ALT73, UNS63 and UNT60 showed least percent mycelia growth of FOL (53.90%), AA (53.68%), CG (56.90%) and RS (53.30%), respectively. Most of the isolates showed percent mycelium inhibition values ranged between 60 and 70% against pathogens. The interaction between pathogens and *Trichoderma* isolates were determined and illustrated by a biplot (Fig. 1). The first two principal component axis of the biplot accounted for 25.54% (PC1) and 27.36% (PC2) of the total variation of the pathogen–antagonist interaction. In this biplot, all the *Trichoderma* isolates were located very far from the origin of biplot, indicating strong antagonism of mycoparasitic isolates towards fungal plant pathogens. Eigen values of the first and second components were 10.508 and 5.471, respectively.

**Production of hydrolytic enzymes**

All *Trichoderma* isolates used in the present study produced cell wall-degrading enzymes (chitinase and β-1, 3 glucanase). Data presented in Table 1 showed that all the mycoparasitic strains produced chitinase and β-1,3 glucanase in the range of 31.0–76.56 μmol GlcNAc min⁻¹ mg⁻¹ protein and 47.67–175.1 nmol glucose min⁻¹ mg⁻¹ protein, respectively. Among all the isolates, maximum chitinase was produced by UNT68, HAT96, UNT38 and UNS30. Similarly, maximum β-1,3 glucanase production was observed in UNS30 followed by DET94 (Table 1). The lowest activities of chitinase (31.00 μmol GlcNAc min⁻¹ mg⁻¹ protein) and β-1,3-glucanases (51.56 nmol glucose min⁻¹ mg⁻¹ protein) were obtained for isolates DET02 and UNT13, respectively. However, most of the isolates showed moderate activities of both lytic enzymes (Table 1).

**Identification of antagonists**

Distinct morphological differences were observed in 5 days old cultures of tested antagonistic isolates grown on PDA (Table 2). A perusal of data indicated that there was a significant difference in growth rate among isolates. Isolates UNT60, UNT68, NAT70, DET89, HAT96, UNT38, UNT13 and UNS30 grew faster (13.3 mm day⁻¹) than other isolates. Less growth rate (11.4 mm day⁻¹) was recorded in case of ALT73 and ALS47 isolates (Table 2). Ellipsoidal and sub-globose to globose conidia were noticed in thirteen isolates (UNT60, UNT64, UNT68, NAT69, NAT70, ALT73, DET89, DET94, HAT96, UNT38, UNS63, UNT09 and DET02). However, it was ellipsoidal and obvoid in rest of the eight isolates (UNT13, UNT70, UNS28, UNS30, NAS46, ALS47 and NAT03). Conidia colour varied from white to watery in all tested isolates. Fourteen isolates (UNT60, UNT64, UNT68, NAT70, DET89, DET94, HAT96, UNS63, UNT13, UNS28, UNS30, NAS46, UNT09 and DET02) showed conidiation concentric zone, while rings were also recorded in six isolates (NAT69, ALT73, UNT38, UNT70, ALS47 and NAT03). Phialides of most of the isolates were tending clustered in 2–3 whorls, but four isolate (NAT69, DET89, DET94 and NAT03) showed solitary disposition (Table 2). The phialides were nine-pin shaped and their size varied between 3.9–13.7 × 1.7–2.9 to 7.0–15.0 × 2.0–3.0 μm in seventeen isolates. However, globose and sigmoid or hooked phialides were also observed in two (UNT68 and ALT73) and one isolate (NAT03), respectively (Table 2).
Molecular identification based on sequences of Tef-1 gene confirmed that the isolates belonged to five different species viz., *T. harzianum* (UNT60, UNT64, UNT68, NAT69, NAT70, ALT73, DET89, DET94 and HAT96), *T. koningii* (UNT38 and UNS63), *T. asperellum* (UNT13 and UNT70), *T. virens* (UNS28, UNS30, NAS46 and ALS47) and *T. viride* (UNT09, DET02 and NAT03) (Table 1). The result of the phylogenetic analysis based on the Tef1 gene sequences of 20 *Trichoderma* isolates is shown in Fig. 2.

**RAPD-PCR analysis**
Five primers viz., OPA-2 (TGCCGAGCTG), OPA-3 (AGTCAGCCAC), OPA-13 (CAGCACCCAC), OPA-15 (TTCCGAACCC) and OPA-18 (AGGTGACCGT) produced a total of 641 fragments among all the 20 isolates (Fig. 3). The size of RAPD fragments ranged 250–2500 bp. Principle component analysis (PCA) showed that RAPD markers explained 31.53% variation among *Trichoderma* isolates at genetic level (Fig. 3). PCA divided the 20 *Trichoderma* isolates in four clusters with pronounced separation of isolates. The first (PCA1) and second (PCA2) principal components were accounted for 20.47 and 11.06%, respectively. Two isolates occupied distinct position, UNT13 was far from the origin while HAT96 was near to the origin of biplot. Cluster I consisted of five isolates (ALT73, NAT03, UNS30, NAS46 and UNT70). However, cluster II comprised nine isolates (NAT70, UNT64, NAT69, ALS47, DET94, UNT38, UNT68, UNT60 and UNS28). Cluster III and IV contained two isolates each.

**BOX-PCR analysis**
BOX-PCR banding pattern showed a total of 200 fragments in the range of 250–4000 bp. The results of PCA analysis based on first and second coordinates showed a maximum Eigen value of 9.306 and minimum value of 0.012 with a percentage variation of 46.53 and 13.72%, respectively (Fig. 4). PCA analysis revealed that nine isolates (UNS28, UNT38, UNT09, DET94, NAT03, NAT69, UNS63, UNT60 and UNT68) formed a major cluster (cluster IV), while three isolates (DET02, UNT70 and UNT13) were grouped in cluster II and two isolates were grouped in Cluster-I (UNS30 and NAS46), VI (DET89 and HAT96) and VII (ALS47 and NAT03).
| Isolate | Colony/PH | Growth rate (mm day\(^{-1}\)) | Colour | Reverse colour | Edge | Mycelial | Conidia | Conidiation | Branching | Shape | Size (µm) | Colour | Phialides |
|---------|-----------|--------------------------|--------|----------------|------|----------|----------|-------------|-----------|-------|----------|--------|-----------|
| UNT60   | *1.33 ± 0.17 | Dark green | Creamish | Wavy | Flocose to Arachnoid | White | Concentric zones | Branched | ellipsodal, subglobose | 1.5–3.4 | Green | Nine-Pin shape | 6–14 x 1.4–2.6 | Tending clustered, 2–3 whorls |
| UNT64   | 1.32 ± 0.17 | Dark green | Creamish | Wavy | Flocose to Arachnoid | Watery white | Concentric zones | Branched | ellipsodal, subglobose | 1.3–3.6 | Green | Nine-Pin shape | 7–15 x 2–3 | Tending clustered, 2–3 whorls |
| UNT68   | 1.33 ± 0.17 | Light green | Light yellow | Wavy | Flocose to Arachnoid | Watery white | Concentric zones | Branched | ellipsodal, subglobose | 1.6–3.0 | Light Green | Globose | 8–14 x 2–3 | Tending clustered, 2–3 whorls |
| NAT69   | 1.16 ± 0.09 | Dark green | Colourless | Wavy | Arachnoid | Watery white | Ring like zones | Branched | ellipsodal, subglobose | 1.7–4.1 | Green | Nine-Pin shape | 6–14 x 14–3 | Solitary |
| NAT70   | 1.33 ± 0.17 | Dark green | Colourless | Smooth | Arachnoid | Watery white | Concentric zones | Branched | ellipsoidal, globose | 1.4–3.7 | Green | Nine-Pin shape | 5.6–14.8 x 2–3 | Tending clustered, 2–3 whorls |
| ALT73   | 1.14 ± 0.12 | Light green | Light yellow | Smooth | Flocose to Arachnoid | White | Ring like zones | Branched | ellipsodal, globose | 1.3–3.3 | Green | Globose | 4.9–11.2 x 1.9–3 | Tending clustered, 2–3 whorls |
| DET89   | 1.33 ± 0.17 | Yellowish green | Light yellow | Wavy | Arachnoid | Watery white | Concentric zones | Branched | ellipsodal, subglobose | 1.5–3.4 | Light Green | Nine-Pin shape | 6–15 x 14–2.8 | Solitary |
| DET94   | 1.15 ± 0.09 | Light green | Creamish | Smooth | Arachnoid | Watery white | Concentric zones | Branched | ellipsodal, subglobose | 1.5–3.4 | Light Green | Nine-Pin shape | 5.9–15.2 x 1.9–2.8 | Solitary |
| HAT96   | 1.33 ± 0.17 | Dark green | Creamish | Smooth | Flocose to Arachnoid | White | Concentric zones | Moderately branched | ellipsoidal, globose | 1.5–3.6 | Green | Nine-Pin shape | 7–14.8 x 19–2.6 | Tending clustered, 2–3 whorls |
| UNT38   | 1.33 ± 0.17 | Light green | Light yellow | Wavy | Arachnoid | Watery white | Ring like zones | Branched | ellipsodal, globose | 1.4–3.8 | Green | Nine-Pin shape | 62–102 x 2.2–2.9 | Tending clustered, 2–3 whorls |
| UNS63   | 1.31 ± 0.16 | Light green | Creamish | Wavy | Flocose | White | Concentric zones | Branched | ellipsodal, globose | 1.5–3.9 | Green | Nine-Pin shape | 58–124 x 2.7–3.2 | Tending clustered, 2–3 whorls |
| UNT13   | 1.33 ± 0.17 | White to green | Light yellow | Wavy | Arachnoid | Watery white | Concentric zones | Branched | ellipsoidal, obovoid | 1.4–3.9 | Dark Green | Nine-Pin shape | 65–117 x 2.7–3.5 | Tending clustered, 2–3 whorls |
| UNT70   | 1.32 ± 0.16 | White to green | Light yellow | Smooth | Flocose to Arachnoid | White | Ring like zones | Moderately branched | ellipsoidal, obovoid | 1.5–3.8 | Light Green | Nine-Pin shape | 61–125 x 2.7–3 | Tending clustered, 2–3 whorls |
Table 2 continued

| Isolate | Colony | Growth rate (mm day$^{-1}$) | Colour | Reverse colour | Edge | Form | Colour | Conidiation | Branching | Shape | Size (µm) | Colour | Shape | Size (µm) | Disposition |
|----------|--------|-----------------------------|--------|----------------|------|------|--------|-------------|-----------|-------|-----------|--------|-------|-----------|-------------|
| UNS28    | 1.31 ± 0.17 | Light green | Creamish | Wavy | Flocose | Watery | white | Concentric zones | Highly branched | ellipsodal, obvoid | 1.4–36 | Light Green | Nine-Pin shape | 5.6–15 × 1.4–3 | Tending clustered, 2–3 whors |
| UNS30    | 1.33 ± 0.17 | Light green | Light yellow | Wavy | Flocose | Watery | white | Concentric zones | Highly branched | ellipsodal, obvoid | 1.3–36 | Green | Nine-Pin shape | 6.8–14.4 × 22–32 | Tending clustered, 2–3 whors |
| NAS46    | 1.16 ± 0.09 | Yellow to green | Light yellow | Wavy | Flocose to Arachnoid | Watery | white | Concentric zones | Branched | ellipsodal, obvoid | 1.4–35 | Green | Nine-Pin shape | 5.5–13.7 × 17–32 | Tending clustered, 2–3 whors |
| ALS47    | 1.14 ± 0.09 | White to green | Light yellow | Wavy | Flocose | Watery | white | Ring like zones | Branched | ellipsodal, obvoid | 1.5–39 | Light Green | Nine-Pin shape | 4.5–12.0 × 17–30 | Tending clustered, 2–3 whors |
| UNT09    | 1.31 ± 0.16 | Dark green | Creamish | Wavy | Arachnoid | White | | Concentric zones | Moderately branched | ellipsodal, subglobose | 1.3–39 | Dark Green | Nine-Pin shape | 3.9–13.7 × 17–29 | Tending clustered, 2–3 whors |
| DET02    | 1.32 ± 0.15 | Yellow to green | Light yellow | Wavy | Flocose | White | | Concentric zones | Moderately branched | ellipsodal, subglobose | 1.5–37 | Green | Nine-Pin shape | 4.5–11.9 × 1.7–2.7 | Tending clustered, 2–3 whors |
| NAT03    | 1.16 ± 0.11 | Light green | Light yellow | Smooth | Flocose | White | | Ring like zones | Branched | ellipsoidal, obvoid | 1.5–3.7 | Green | Sigmoid or hooked | 5–12 × 2.2–2.7 | Solitary |

* Within columns, mean ± SE values with a common letter do not differ significantly (P < 0.05), according to DMRT test
Fig. 2 Neighbor joining tree (Kimura two-parameter distance) of twenty *Tef*-1a sequences of *Trichoderma* isolates from tomato rhizosphere. The numbers given over branches indicate bootstrap coefficient.
ERIC-PCR analysis
The genetic discrimination among the 20 isolates was assessed using ERIC-PCR and a high level of variability in the banding pattern was obtained (Fig. 5). The number of bands in the amplification profile was 182, and their size was found to vary from 250 to 3000 bp among these isolates (Fig. 5). Principal component analysis (PCA) based on first and second coordinates showed a maximum Eigen value of 10.027 and minimum value of 0.01 with a percentage variation of 50.13 and 12.09, respectively (Fig. 5). A perusal of the PCA analysis revealed that eight isolates (HAT96, UNT68, DET94, UNT60, UNT64, NAT69, DET02, DET89 and UNT09) formed a major cluster (cluster IV), while three isolates were grouped in cluster II (UNS28, UNS63 and UNT38) and IV (UNT70, NAT03 and ALT73).

REP-PCR analysis
The genetic discrimination among the 20 isolates was assessed using REP-PCR and a high level of variability in the banding pattern was obtained (Fig. 6). The number of bands in the amplification profile was 350, and their size was found to vary from 270 to 3000 bp among these isolates (Fig. 6). Principal component analysis (PCA) based on first and second coordinates showed a maximum Eigen value of 9.758 and minimum value of 0.017 with a percentage variation of 48.71 and 13.16, respectively (Fig. 6). A perusal of the PCA analysis revealed that six isolates (UNS30, NAS46, NAT03, ALS47, UNS28 and UNT70) formed a major cluster (cluster V), while four isolates were grouped in cluster VI (UNT13, DET02, UNT09 and UNS63).

ERG1 sequencing and phylogenetic analysis
Detection of squalene epoxidase (ERG1) gene in Trichoderma isolates was shown in Fig. 7. Squalene epoxidase (ERG1) gene amplification showed one specific band (500 bp) in all the twenty Trichoderma isolates. The phylogenetic tree obtained by sequence analysis of ERG1 region of all the tested isolates is represented in Fig. 8. A neighbour-joining analysis of the alienable ERG1-sequences of all the tested isolates demonstrated two distinct phylogenetic clades. Clade A comprised mainly T. harzianum (UNT60, UNT64, UNT68, NAT69 and UNT70), T. viride (UNT09, DET02 and NAT03), T. koningii (UNS63) and T. virens (UNS28) and showed very
high homology to the nearest ERG1 sequence of H. lixii, T. arundinaceum and T. reesei submitted in NCBI GenBank. Clade B represented four isolates of T. harzianum (ALT73, DET89, HAT96, and DET94), two isolates of T. koningii (UNT38), two isolates of T. asperellum (UNT13 and UNT70) and three isolates of T. virens (ALS47, UNS30 and NAS46) and showed heterogeneity with respect to the ERG1 sequence of H. lixii, T. arundinaceum and T. reesei.

Discussion

Microbial inoculants with antagonistic properties towards fungal plant pathogens have a potential to replace chemical pesticides since they are known for growth promotion and disease reduction in crops. Several species of Trichoderma have been used as biological control agents to manage diseases of vegetable and other crops (Solanki et al. 2011; Srivastava et al. 2012; Al-Sadi et al. 2015). In the present study, twenty isolates of Trichoderma collected from rhizosphere soil of tomato were phenotypically, biochemically and genetically characterized to identify and screen the most efficient antagonistic against four tomato fungal pathogens (FOL, AA, CG and RS). All the tested isolates grew considerably faster than the fungal pathogens and quickly controlled the pathogens. The ability to grow rapidly gives antagonists an important advantage in competition for space and nutrients with pathogen (Benitez et al. 2004; El_Komy et al. 2015). Nine isolates (UNT68, DET94, HAT96, UNT38, UNS30, DET02, ALS47, UNS28 and UNT09) showed significant percent mycelium inhibition against the test pathogens. These isolates overgrew and sporulated on the pathogen colonies. In the interaction zone, the mycelia of all the fungal pathogens had abnormal morphology and lysed, which implies the occurrence of strong mycoparasitism. These results are in conformity with previous studies where Trichoderma isolates showed high capabilities as versatile biocontrol agents (Trillas et al. 2006; Tondje et al. 2007; de los Santos-Villalobos et al. 2013). Interestingly, the interaction of indigenous Trichoderma isolates with four different fungal pathogens resulted in significantly different amounts of pathogen inhibition. For instance, DET94 had very strong inhibitory effect on the growth of FOL, RS and CG pathogens,
whereas moderate inhibition effect was recorded in case of AA. These results are consistent with the findings of Markovich and Kononova (2003). They reported that the mycoparasitic capacity of various species and isolates of *Trichoderma* differs. There are several mechanisms involved in *Trichoderma* antagonism, namely, antibiosis whereby the antagonist fungus produces antibiotics, competes for nutrients and mycoparasitism, whereas *Trichoderma* directly attacks the plant pathogen by excreting lytic enzymes such as chitinases and β-1,3 glucanases (Kubicek et al. 2001; Rajacommare et al. 2010; Solanki et al. 2011). Such hydrolytic enzymes partially degrade the pathogen cell wall that leads to parasitization (Howell 2003). Also in the present study antagonistic isolates (UNT68, DET94, HAT96, UNT38, UNS30, DET02, ALS47, UNS28 and UNT09) with the highest levels of enzyme activities showed the strong inhibitory effect on the growth of fungal plant pathogens. Similar observations were made by Howell (2003), wherein the activity of lytic enzymes (chitinases and β-1,3 glucanases) was responsible for lysis of *R. solani* hyphae through digestion of major cell wall components. There was a positive relationship between the antagonistic capacity of the *Trichoderma* isolates and the production of chitinase and β-1,3-glucanases. Thus, efficient antagonistic isolates inhibited fungal growth through the production of lytic enzymes. On parallel lines, Lopes et al. (2012) reported a positive correlation between the lytic enzymes activities and the antagonism capacity of *T. asperellum* against *Sclerotinia sclerotiorum*. Moreover, Qualhato et al. (2013) and El_Komy et al. (2015) reported that there was a positive correlation between the amounts of secreted cell-wall degrading enzymes by *Trichoderma* strains and their ability to control plant pathogenic fungi.

Taxonomic knowledge on *Trichoderma* isolates is important for identification and characterization of potential biocontrol species and to avoid potential risk from introducing an unknown fungal species into the rhizosphere of a given ecosystem. A combination of morphological and molecular methods is desirable for the reliable and accurate identification of *Trichoderma* spp. The few morphological characteristics with limited variation in *Trichoderma* spp. may lead to an overlap and wrong identification of the species (Galarza et al. 2015). In present study, *Trichoderma* isolates were categorized on the basis of description and keys given by Gams and...
Bissett (1998). As a result, ellipsoidal and sub-globose to globose condial structures resembled with \textit{T. harzianum}, while ellipsoidal and ovoid shaped conidia were matched with \textit{T. virens} isolates; as previously mentioned by Choi et al. (2003). However, some isolates showing overlapping characters and resembling with \textit{T. koningii}, \textit{T. viride} and \textit{T. asperellum} could not be separated using the morphology-based method. Thus, molecular identification of
Trichoderma isolates at the species level was done on the basis of TEF-1a gene as it has been reported to be better for distinguishing Trichoderma spp. (Samuels 2006).

The present study also revealed the usefulness of DNA polymorphism techniques to detect genetic variation among antagonistic Trichoderma isolates. These techniques are important not only for understanding their ecological role in the rhizosphere, but also to characterize the biological control agents for registration and patenting biocontrol strains, recognizing the strains, quality checking during production and ecological characterization (Plimmer 1993). The study of DNA polymorphisms involves the selection of a target sequence, and several approaches have been used to achieve this task. One approach involves the exploitation of ubiquitously conserved known genes that display sequence variation. Identification of Trichoderma to the species level based on reference sequences from the National Center for Biotechnology Information correlated with phylogenetic analysis based on sequences of the ITS rRNA and the translation elongation factor gene (EF1a). However, the limited intraspecific variation within Trichoderma species based on sequences of the EF1a gene helped giving better resolution in separating Trichoderma species when compared to sequences of the ITS region (Al-Sadi et al. 2015). Thus, in present study, comparative nucleotide sequencing of EF1a gene was performed to distinguish and identify antagonistic Trichoderma isolates. Based on the sequence analysis of EF1 gene, the 20 antagonistic isolates were divided in five species: T. harzianum, T. koningii, T. asperellum, T. virens and T. viride. Another approach involves the screening of random parts of the genome to identify distinctive nucleotide sequences by techniques, such as RAPD, REP-, ERIC- and BOX-PCR.
The results indicated that BOX elements and ERIC-PCR are suitable for the rapid genetic differentiation of *Trichoderma* isolates. Some of the *Trichoderma* isolates such as NAT70, UNT64 and ALS47 which were not differentiated by RAPD can be discriminated by BOX and ERIC-PCR banding patterns. In general, both techniques were found to produce reproducible results especially with purified genomic nucleic acid as a template, and when the primer concentration and composition of buffer were strictly controlled. It is also worth mentioning here that ERIC-, REP- and BOX-PCR marker systems revealed >60% intra-species variability among *Trichoderma* isolates, although clustering on the basis of antagonism, geographical origin and hydrolytic enzyme production was not detected. Additionally, the present study was unable to correlate biomarker variation with fungal growth inhibition activity of *Trichoderma* isolates. These findings are in agreement with earlier studies, where no defined correlations between genetic variability assessed by random markers (e.g. RAPD) and the ability of *Trichoderma* isolates to inhibit fungal mycelia growth were obtained (Sharma et al. 2009; El_Komy et al. 2015). This may be due to the ubiquitous nature and seemingly random chromosomal distribution of random repeats in *Trichoderma* genome, giving rise to simultaneous PCR amplification of multiple genomic regions (Rai et al. 2016). The high genotypic variability among *Trichoderma* isolates could be associated with mutations in priming sites, rearrangements of chromosomal segments or recombination process in fungal genomes (Kumar et al. 2012, 2013b). However, genetic variability among *Trichoderma* isolates in addition to their differences in fungal growth inhibition toward fungal plant pathogens suggest that combinations of isolates could further be applied in both greenhouse and field studies to manage tomato diseases.

Terpene compounds (e.g., ergokonins and viridins) are involved in the biocontrol process due to their antifungal properties (Malmierca et al. 2015). Similar to this, the present study also documented the possibility of squalene epoxidase driven triterpene biosynthesis mechanism in biocontrol of tomato wilt and foliar blight diseases. Furthermore, PCR based detection of *ERG1* gene in antagonistic isolates confirmed the presence of gene at molecular level and Blastn and Blastp results showed the maximum homology with a squalene epoxidase gene. Phylogenetic analysis of squalene epoxidase gene (*ERG1*) sequences revealed close relatedness of *ERG-1* sequences with earlier reported sequences of *H. lini*, *T. arundinaceum* and *T. reesei*. However, *ERG1* gene also showed heterogeneity among some antagonistic isolates and it may be possible that squalene epoxidase driven triterpene biosynthesis have an important role in biocontrol mechanisms of tested isolates.

In conclusion, the present study provides preliminary information on the biological control of tomato diseases by correctly identifying the fungal antagonists. Correct identification will provide information on understanding the interparasitic relationship with target pathogens and the subsequent environmental fate of the antagonist needed for effective application. Further, combined studies including biological, biochemical and molecular technologies, are essential to select indigenous antagonistic *Trichoderma* isolates that can be used under different environmental conditions. Genetic variability of squalene epoxidase (*ERG1*) gene among these isolates in addition to their differences in aggressiveness toward multiple fungal pathogens suggest that combinations of isolates could further be applied in both greenhouse and field studies to obtain resistance against multiple fungal pathogens in tomato crop. However, further experiments are needed to validate the role of squalene epoxidase driven triterpene biosynthesis in biocontrol mechanisms of tested isolates.

**Authors’ contributions**
SR carried out the sampling and performed assays for the screening of antagonistic *Trichoderma*. PLK participated in the design of the study, performed statistical analysis and helped to draft the manuscript. SK participated in the identification of *Trichoderma* isolates, sequence alignment and drafted the manuscript. AKS carried out the molecular characterization of antagonists and sequence submission. PWR conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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**Competing interests**
The authors declare that they have no competing interests.

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