Ann Microbiol (2014) 64:1513–1522
DOI 10.1007/s13213-013-0794-7

ORIGINAL ARTICLE

Characterization of *Trichoderma* spp. antagonistic to *Phytophthora colocasiae* associated with leaf blight of taro

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Received: 14 July 2013 / Accepted: 18 December 2013 / Published online: 5 January 2014

Abstract Eighteen *Trichoderma* strains isolated from soils of different regions of India were analyzed for their biocontrol potential against the taro leaf blight pathogen *Phytophthora colocasiae*. The strains were characterized using a combination of phenotypic and molecular approaches, viz., in vitro antagonism, extracellular enzyme production, ITS and *tef1* gene sequencing and AFLP analysis. Eight isolates exhibited more than 75 % inhibition of the pathogen *P. colocasiae*. Differential antagonistic activity was evident for different strains. Assay of lytic enzyme production suggested the role of glucanase in mycoparasitism of *P. colocasiae*. Sequencing and phylogenetic analysis of the *Trichoderma* strains showed that isolates belong to three species: *Trichoderma asperellum*, *Trichoderma longibrachiatum* and *Trichoderma harzianum*. AFLP analysis revealed profound genetic diversity among the isolates. The most potent strain overall (TR7) identified as *T. harzianum* was able to control *P. colocasiae* in vivo also. This isolate could be used as a promising candidate for biological control of taro leaf blight disease.

Keywords Biological control · *Colocasia esculenta* · Disease management · *Trichoderma asperellum* · *Trichoderma harzianum* · Oomycetes

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Introduction

Taro [*Colocasia esculenta* (L.) Schott] is an important tropical tuber crop, used as a staple or subsistence food by millions of people in developing countries in Asia, Africa and Central America. Leaf blight caused by the oomycetes pathogen *Phytophthora colocasiae* Raciborski is the most destructive disease affecting taro causing yield losses to the extent of 30–50 % every year (Misra et al. 2008).

Several approaches are used to combat the disease including crop rotation and the use of fungicides. Despite the effectiveness of fungicides the presence of a waxy coating on the leaf lamina makes it ineffective. Also, development of resistance to fungicide is another major threat (Cohen and Coffey 1986; Lambert and Salas 1994; Gisi and Cohen 1996). Buildup of metalaxyl resistance has already been demonstrated in field isolates of *P. colocasiae* (Nath et al. 2012a). Recent studies have shown that *P. colocasiae* has a high genotypic diversity which suggests a high potential to develop new virulent strains able to overcome plant resistance (Lebot et al. 2003; Nath et al. 2012b). Thus, the need for finding an alternative to manage leaf blight disease in taro production systems is acute.

Biocontrol of plant pathogens has attracted significant attention as an alternative disease management strategy because of its ability to provide environmentally friendly disease control. Several potential biocontrol organisms have been isolated, characterized and commercialized. These include *Trichoderma* species (Harman 2006); mycoparasitic species of the genus *Verticillium* (Fenice et al. 1998); rhizobacteria in the genera *Pseudomonas*, *Bacillus*, *Streptomyces* (Weller 2007; Fróes et al. 2012; Ashwini and Srividya 2013); *Lecanicillium* species (Fenice and Gooday 2006). However, among the microorganisms reported as biocontrol agents, *Trichoderma* species have gained significant attention because of their multiprong action against several economically important plant pathogens (Harman et al. 2004). Several

mechanisms responsible for inhibition of the pathogen by *Trichoderma* spp. has been described such as competition for nutrients and space, mycoparasitism, antibiosis, promotion of plant growth and plant defense responses, and production of antifungal compounds (Haran et al. 1996; Harman et al. 2004). However, the process termed mycoparasitism, has been proposed as the major mechanism accounting for antagonistic activity against the fungal pathogens where the antagonist coils around the pathogen coupled with the production of lytic enzymes (Howell 2003; Harman et al. 2004).

Understanding the mechanisms of interaction of *Trichoderma* spp. with the plant pathogen and plant host is a prerequisite to their effective practical application. Moreover, understanding the nature and diversity of biocontrol agents is critical for its potentially widespread use for control of phytopathogenic fungi as there could be the risk of unwanted disease on non-target hosts (Jeffries et al. 1994).

Trichoderma spp. has been reported as limiting *Phytophthora* diseases of crop plants. Potential *Trichoderma* spp. discovered include *Trichoderma harzianum* against *P. capsici* (Ahmed et al. 1999), *T. harzianum* and *T. virens* against *Phytophthora erythroseptica* (Etebarian et al. 2000), and *Trichoderma* spp. against *Phytophthora palmivora* (Mpika et al. 2009). However, the role of *Trichoderma* spp. in controlling *P. colocasiae* causing leaf blight of taro is not well understood. To the best of our knowledge no extensive study has been reported concerning the characterization of *Trichoderma* spp against *P. colocasiae* causing leaf blight of taro.

The goals of the research reported here was (1) to isolate *Trichoderma* spp. from rhizosphere soils of different regions of India and (2) to characterize these isolates through a polyphasic approach using morphological, biochemical and molecular methods.

Materials and methods

Fungal isolates

The study was conducted with 18 strains of *Trichoderma* spp. isolated from taro rhizosphere soil samples originating from different regions of India (Table 1). The *Trichoderma* spp. were isolated from 1 g soil sample suspended in 10 ml sterile distilled water. Aliquots of serially diluted samples were spread on potato dextrose agar (PDA; 250 g l^{-1} potato, 20 g l^{-1} dextrose and 20 g l^{-1} agar) containing 0.1 mg ml^{-1} ampicillin and incubated at 28 °C in low light conditions for 6

Table 1 Details of *Trichoderma* isolates used in this study along with their in vitro antagonistic activity against *P. colocasiae* determined using dual culture, production of diffusible and volatile inhibitors on PDA plates. Plates were incubated at 28 °C for 6 days

Strain	Place of isolation	Percentage of inhibition of *P. colocasiae* growth		
		Dual culture	Diffusible inhibitors	Volatile inhibitors
TR1	Vencode, Kerala	55.76±0.14^{d}	50.66±0.08^{e}	0.00±0.00^{a}
TR2	Bomdilla, Arunachal Pradesh	74.83±0.16^{f}	58.60±0.20^{g}	5.06±0.06^{b}
TR3	Munnar, Kerala	76.00±0.11^{g}	41.76±0.14^{c}	12.36±0.20^{g}
TR4*	Salem, Tamil Nadu	86.33±0.17^{k}	72.93±0.06^{j}	14.90±0.10^{i}
TR5*	Bhubaneswar, Odisha	78.70±0.11^{h}	42.53±0.03^{d}	0.00±0.00^{a}
TR6*	Jorhat, Assam	100.00±0.00^{n}	77.60±0.05^{l}	13.36±0.08^{h}
TR7*	Nelliampathy, Kerala	100.00±0.00^{n}	100.00±0.00^{o}	30.30±0.05^{m}
TR8*	Kanjirampally, Kerala	98.16±0.08^{m}	78.23±0.08^{m}	10.40±0.11^{f}
TR9	Munnar, Kerala	81.50±0.05^{i}	74.90±0.05^{k}	9.93±0.06^{e}
TR10*	Kollam, Kerala	87.13±0.08^{l}	71.43±0.12^{h}	8.50±0.05^{d}
TR11*	Idukki, Kerala	84.90±0.05^{j}	71.83±0.08^{i}	7.16±0.12^{c}
TR12	Puri, Oidsha	55.96±0.08^{d}	53.60±0.05^{f}	0.00±0.00^{a}
TR13	Parudin palam, Andhra Pradesh	46.36±0.20^{c}	20.46±0.08^{b}	0.00±0.00^{a}
TR14*	CTCRI, Kerala	100.00±0.00^{n}	79.63±0.08^{n}	17.46±0.14^{k}
TR15*	CTCRI, Kerala	100.00±0.00^{n}	78.46±0.14^{m}	15.56±0.03^{j}
TR16	Ribhoi, Meghalaya	44.93±0.06^{b}	0.00±0.00^{a}	0.00±0.00^{a}
TR17	Kaveri, Andhra Pradesh	39.66±0.43^{a}	0.00±0.00^{a}	0.00±0.00^{a}
TR18	Bangaluru, Karnataka	63.13±0.17^{e}	42.30±0.11^{d}	22.16±0.12^{l}

*Isolates used for molecular characterization

Values are the mean±SE of three replicates. Mean values in the same column followed by same letter are not significantly different according to Duncan's multiple range test at $P \leq 0.05$

days. Fungal colonies with the characteristics of *Trichoderma* such as fast growth, green colonies, and formation of concentric green and white rings were selected, and the genus was confirmed microscopically. All strains were maintained throughout the study by periodical transfers on PDA medium under aseptic condition to keep the culture fresh and viable. For long term storage, the isolates were stored in 50 % glycerol at -20 ° C.

A highly virulent isolate of *P. colocasiae* (P21), obtained from leaf blight infected samples maintained by Central Tuber Crops Research Institute (CTCRI), Thiruvananthapuram, India, was used for the study. The authenticity of the isolate was confirmed by ITS amplification and sequencing (Nath et al. 2013).

Assessment of antagonistic activity in vitro

Trichoderma isolates were evaluated for their potential to antagonize the leaf blight pathogen *P. colocasiae* using three different tests in vitro. For all tests discs of 5 mm diameter were excised from the margins of actively growing cultures of the antagonist and the pathogen. All incubations were performed in the dark.

In the first test, the *Trichoderma* spp. were evaluated for their antagonistic activity against the pathogen by dual culture assay on PDA (Dennis and Webster 1971c). The *P. colocasiae* isolate (P21) and the *Trichoderma* isolates were inoculated 6 cm apart on the same plate and incubated at 28 °C for 6 days. Control plates were seeded with sterile agar plugs in place of antagonist. Following incubation, radial growth of pathogen, *P. colocasiae* was measured and the percent inhibition of average radial growth was calculated relative to the control (Edington et al. 1971).

The second test assessed the ability of *Trichoderma* isolates to produce diffusible inhibitors against *P. colocasiae*. *Trichoderma* agar discs were placed on the centre of PDA plates covered with a cellophane membrane and incubated for 6 days at 28 °C. Following incubation, the cellophane membranes were asceptically removed and *P. colocasiae* was cultured in the same plates (Dennis and Webster 1971a). Growth of *P. colocasiae* was recorded at 6 days of incubation. Plates without the prior inoculation of *Trichoderma* isolate under same conditions mentioned above served as the control experiment.

The third test measured the ability of *Trichoderma* isolates to produce volatile inhibitors. The PDA plates were centrally inoculated with agar discs of *Trichoderma* isolates and the lid of each dish was replaced by a bottom dish containing PDA inoculated with an agar disc of *P. colocasiae*. The two dishes were sealed together with adhesive tape (Dennis and Webster 1971b) and incubated at 28 °C. The growth of *P. colocasiae* was recorded after 72 h of incubation. In the control plate, *P. colocasiae* was cultured in the same manner but without *Trichoderma* isolates.

A completely randomized design was used and all experiments were performed with three independent replicates and the experiments were repeated at least twice.

Extracellular enzyme production

Trichoderma isolates were grown on PDA plates at 28 °C in the dark. A 100 ml of minimal synthetic medium supplemented with 0.5 % colloidal chitin was inoculated with three agar discs (5 mm) of *Trichoderma* (Sharma et al. 2009). The seeded flasks were incubated at 150 rpm on a rotary shaker at 28 °C in the dark for 7 days for the production of lytic enzymes. Culture filtrates were centrifuged at 5,000×*g* for 10 min at 4 °C and stored at -20 °C until further use.

Chitinase (EC 3.2.1.14) activity was assayed using the colorimetric method as described by (Molano et al. 1977) with minor modifications. The assay mixture contained 1 ml of 0.5 % colloidal chitin (suspended in 50 mM acetate buffer pH 5.2) and 1 ml of enzyme solution. The reaction mixture was incubated for 1 h at 40 °C with shaking. The reaction was stopped by placing the vials into a boiling water bath for 5 min and then 1 ml of dinitrosalicylate was added to the reaction mixture. The amount of reducing sugars released was calculated using a standard curve recorded for N- acelylglucosamine (GlcNAc). One picokatal (pkat) of enzyme activity releases one pmol s^{-1} of GlcNAc equivalent under the specific assay conditions.

Similarly, β -1,3 glucanase (EC 3.2.1.3.9) was assayed by incubating 1 ml of enzyme solution to 2 ml of 5 mg ml^{-1} laminarin (50 mM acetate buffer, pH 4.8) at 50 °C for 1 h and determination of glucose with dinitrosalicylate. One nanokatal (nkat) of β-1,3-glucanase activity was defined as the amount of enzyme that releases one nmol s^{-1} of glucose equivalent under the specific assay conditions.

Rapid in vivo assay

The *Trichoderma* strain (TR7) presenting the highest biocontrol ability against *P. colocasiae* in vitro, was tested on detached taro leaves to study its biocontrol capacity in limiting the pathogen in vivo. For the rapid in vivo assay, taro leaves (cv. Sree Kiran, leaf blight susceptible) were disinfected by immersing in 1 % sodium hypochlorite solution for 1 min, rinsed twice with sterile double distilled water and blotted dry on sterile paper towels. Leaf discs (5×5 cm) were floated on *Trichoderma* mycelial water suspension (scraped from 20 day old PDA plate grown at 28 °C in the dark) in 90-mm Petri plates and inoculated with a mycelial disc (5 mm) excised from the margins of actively growing cultures of *P. colocasiae*. Leaf discs with *P. colocasiae* agar plug on sterile distilled water served as control treatment. Plates were covered with

lid containing moistened filter paper to maintain high humidity and incubated in a moist chamber at 28 °C in the dark for 4 days. Following incubation the disease symptoms were assessed visually and subsequently the lesion diameter was estimated. Each assay consisted of five replications and the assay was repeated twice at same conditions mentioned above.

Molecular identification and characterization

The promising isolates of *Trichoderma* spp. determined by in vitro assay were identified to the species level by DNA sequencing of two regions, the ribosomal ITS region and the *tef1* gene (Table 1). The ITS region was amplified using the universal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al. 1990). Each 25 μL of PCR reaction consisted of 50 ng of template DNA, 100 μM each deoxynucleotide triphosphate, 20 ng of each primer, 1.5 mM $MgCl_2$, 1 × Taq buffer (10 mM Tris–HCl pH 9.0, 50 mM KCl, 0.01 % gelatin), 1 U of Taq DNA polymerase (Merck GeNei, India). Amplifications were performed in an Agilent sure cycler 8800 (Agilent Technologies, USA). The thermal cycler was programmed as follows: 2 min at 94 °C, 35 cycles of 30 s at 94 °C, 1 min at 57.1 °C and 1 min 30 s at 72 °C, and finally 8 min at 72 °C. Amplified products were resolved on a 1.5 % agarose gel containing 0.5 μg ml^{-1} ethidium bromide and photographed through the Gel Doc System (Alpha imager, Alpha Innotech, USA).

The *tef1* fragment was amplified by using the primers EF1-728 F (5′-CATCGAGAAGTTCGAGAAGG-3′) and EF1-986R (5′- TACTTGAAGGAACCCTTACC-3′) (Druzhinina et al. 2005; Jaklitsch et al. 2006) under the same PCR conditions stated above with the exception of the following parameters that differed: the temperature of annealing (48 °C for 30 s) and extension (72 °C for 1 min). The PCR products were checked by electrophoresis and images were acquired as mentioned above.

The ITS and *tef1* amplicons were purified from agarose gel using a QIAquick Gel extraction kit (QIAGEN, Tokyo, Japan) and sequenced in both directions using the same primers as for amplification. Sequencing was carried out in the DNA fingerprinting wing of Rajiv Gandhi Centre for Biotechnology (RGCB), Thiruvananthapuram. The nucleotide sequences obtained were quality checked, processed to remove indels and transformed into consensus sequences with Geneious Pro software version 5.6. Sequence identities were determined by using Genbank database and a specific database for *Trichoderma*, International Subcommission on *Trichoderma* and *Hypocrea* (ISTH, www. isth.info). All sequences obtained were deposited in the NCBI Genbank database.

Consensus sequences of the ITS1 and *tef1* region of *Trichoderma* strains and reference sequences downloaded from Genbank were aligned using the multiple sequence alignment program Clustal W and a phylogenetic study was performed using the program MEGA version 5 (Tamura et al. 2011). The analysis was conducted using the unweighted pair group method with the arithmetic mean (UPGMA) method. The evolutionary history was inferred using the neighbor-joining method (Saitou and Nei 1987) and the evolutionary distances were computed using the maximum composite likelihood method. The robustness of the internal branches was assessed with 2,000 bootstrap replications.

AFLP analysis

AFLP analysis was performed with the promising isolates identified from in vitro confrontation assay as previously described (Nath et al. 2012b). After pre-screening 35 primer pairs, six selective primer pairs were chosen for this study (Table 2).

All clearly detectable AFLP bands were scored for their presence (1) or absence (0) by visual observation. In order to ensure credibility only reproducible and well defined bands were scored. A dendrogram was constructed using genetic similarity matrices to display relationships between isolates using the Nei and Li (1979) according to the unweighted pair group mean algorithm using the 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 (2,000 replicates). The cophenetic correlation coefficient was calculated to provide statistical support for the dendrogram obtained, and Mantel's test (Mantel 1967) was performed to check the goodness-of-fit of the cluster analysis of the matrix on which it was based (1,000 permutations).

Statistical analysis

The data on percentage inhibition of *P. colocasiae* and extracelluar enzyme assays were analyzed by analysis of variance (ANOVA) and the means were compared by Duncan's Multiple Range Test (DMRT) using SPSS (version17.0; SPSS, Inc., Chicago, IL, USA). The percentage values were arc sin transformed before analysis. Statistical significance was defined as $P \leq 0.05$.

Results

Assessment of antagonistic activity in vitro

Three independent tests such as dual culture, production of diffusible inhibitors and production of volatile inhibitors were adopted to screen the isolates. The results showed that the majority of *Trichoderma* strains had high antagonistic potential against the pathogen *P. colocasiae* compared to the

Table 2 Molecular identification of selected *Trichoderma* isolates based on ITS and *Tef1* gene sequencing

Strain	*Tef 1* sequence				ITS sequence				Definitive identification	Genbank accession no.	
	Fasta analysis	Fasta identity (%)	*Trichoderma* ISTH database	Tricho blast identity (%)	Fasta analysis	Fasta identity (%)	*Trichoderma* ISTH database	Tricho blast identity (%)		ITS sequence	*Tef 1* sequence
TR4	*Trichoderma asperellum* (JF964995)	99	*Trichoderma asperellum* (CBS433.97)	89	*Trichoderma asperellum* strain GDFS1009 (JQ617295)	99	*T.theobromicola* (DIS85f)	98	*Trichoderma asperellum*	KC859425	KC572115
TR5	*Trichoderma longibrachiatum* (EU280046)	99	*Trichoderma longibrachiatum* (GJS88-81)	98	*Trichoderma longibrachiatum* (KC009811)	100	*Trichoderma longibrachiatum* (ATCC18648)	100	*Trichoderma longibrachia-tum*	KC859426	KC572116
TR6	*Trichoderma asperellum* (JF964995)	99	*Trichoderma asperellum* (CBS433.97)	89	*Trichoderma asperellum* strain LT89 (HQ392484)	100	*T.theobromicola* (DIS85f)	99	*Trichoderma asperellum*	KC859427	KC572117
TR7	*Hypocrea lixii* strain DAOM 231435 (EF191321)	99	*Trichoderma harzianum* (GJS92-61)	90	*Hypocrea lixii* (AB563725)	99	*Trichoderma harzianum* (DAOM231412)	99	*Trichoderma harzianum*	KC859428	KC572118
TR8	*Trichoderma asperellum* (JF964995)	99	*Trichoderma asperellum* (CBS433.97)	90	*Trichoderma asperellum* isolate AN1 (GQ451697)	100	*T.theobromicola* (DIS85f)	95	*Trichoderma asperellum*	KC859429	KC572119
TR10	*Trichoderma asperellum* strain T34 (EU077228)	99	*Trichoderma asperellum* (CBS433.97)	99	*Trichoderma asperellum* clone 167 (JQ973613)	99	*T.theobromicola* (DIS85f)	98	*Trichoderma asperellum*	KC859431	KC572121
TR11	*Trichoderma asperellum* (JF964995)	99	*Trichoderma asperellum* (CBS433.97)	91	*Trichoderma asperellum* strain AF35 (JX677935)	98	*H.flaviconidia* (GJS99-49)	97	*Trichoderma asperellum*	KC859432	KC572122
TR14	*Trichoderma asperellum* (JF964995)	99	*Trichoderma asperellum* (CBS433.97)	89	*Trichoderma asperellum* strain AF14 (JX677934)	99	*T.theobromicola* (DIS85f)	99	*Trichoderma asperellum*	KC859433	KC572123
TR15	*Trichoderma asperellum* (JF964995)	99	*Trichoderma asperellum* (CBS433.97)	89	*Trichoderma asperellum* strain AF35 (JX677935)	99	*T.theobromicola* (DIS85f)	99	*Trichoderma asperellum*	KC859434	KC572124

control. *Trichoderma* isolates TR6, TR7, TR14, TR15 exhibited maximum inhibition of *P. colocasiae* in dual cultures. In contrast, TR13, TR16 and TR17 showed the lowest rate of inhibition (40–46 %). The remaining isolates shared intermediate values of the percentage of inhibition of *P. colocasiae* growth (Table 1).

The results of the test to produce diffusible inhibitors are also presented in Table 1. The isolate TR7 completely inhibited the growth of *P. colocasiae*, followed by TR6, TR8, TR14, and TR15 which showed high inhibition potential (approximately 78 %). The remaining isolates were poor producers of diffusible metabolites.

The inhibition of *P. colocasiae* growth by volatile inhibitors was not very remarkable. The highest inhibitory activity was shown by TR7 (30.3 %). The remaining isolates exhibited no or poor production of volatile inhibitors (Table 1).

Extracellular enzyme production

Based on the in vitro assessment of antagonistic activity, we selected nine *Trichoderma* isolates to measure their extracellular enzyme production. The chitinase (EC 3.2.1.14) and β-1,3 glucanase (EC 3.2.1.3.9) activities of the isolates are summarized in Fig. 1. In general, the isolates that showed a greater inhibitory effect on radial growth of *P. colocasiae* also produced high levels of enzymes in the assay. There was a significant difference in the amount of enzyme secreted by individual isolates. The highest level of β -1,3 glucanase (EC 3.2.1.3.9) production was observed in isolate TR7, while the maximum level of chitinase (EC 3.2.1.14) production was observed in isolate TR10.

Rapid in vivo assay

The *Trichoderma* strain TR7 which showed the highest antagonistic activity in vitro was also efficient in restricting disease development in taro leaf discs when compared to the control. During treatment with *Trichoderma* strain TR7, the disease severity was reduced by more than 50 % (mean lesion diameter of 1.2±0.02) when compared to the control treatment (mean lesion diameter of 3.2±0.3), after 4 days of incubation.

Molecular identification and characterization

All isolates were successfully amplified using PCR and produced an amplicon of approx. 600 bp for the ITS region and approximately 300 bp for the *tef1* region. The obtained sequences from the two regions were individually queried against the NCBI database and the ISTH database to identify the isolates to the species level. The *Trichoderma* strains depicted the high degree of similarity (89–100 %) to reported sequences in the database. Three species were identified, viz., *Trichoderma harzianum*, *Trichoderma longibrachiatum* and *Trichoderma asperellum* (Table 2). In some instances, the sequences produced ambiguous results upon the BLAST analysis using NCBI and ISTH databases and; therefore, in these cases species appearing maximum number of time was used as a source for definitive identification of the isolates.

The phylogenetic analysis complemented the results of molecular identification. The phylogenetic tree clearly portrayed the relationships among the *Trichoderma* isolates. There was detectable variation among the *Trichoderma* isolates, which is evident from the dendrogram. The isolates were successfully clustered to their respective species groups (in both cases, i.e., ITS and *tef1* gene) based on their degree of similarity with high bootstrap values, indicating the robustness of the clustering. The phylogram generated based on the *tef1* region is shown in Fig. 2.

AFLP analysis

AFLP analysis produced a large number of reproducible and unambiguous markers for fingerprinting the isolates of *Trichoderma* spp. The bands were distinct and easy to score. Six *Eco*RI+2/*Taq*I+2 primer pair combinations resolved 406 markers that could be scored reliably. A high percent of polymorphism (94.4 %) was observed across the eight isolates

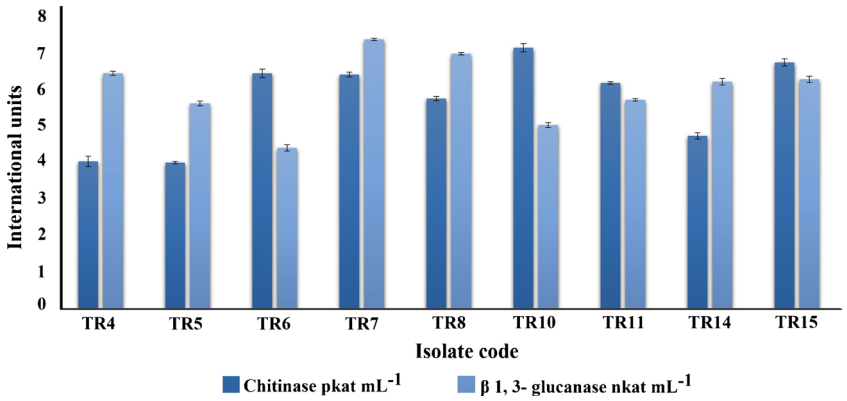

Fig. 1 Chitinase and glucanase activities of promising *Trichoderma* strains selected based on an in vitro antagonistic assay. The activities of chitinase and β -1,3 glucanase were expressed in pmol GlcNAc released per second (pmol s^{-1}) and nano mol glucose released per second (nmol s^{-1}), respectively, under the specific assay conditions. Values are the mean± SE of three replicates

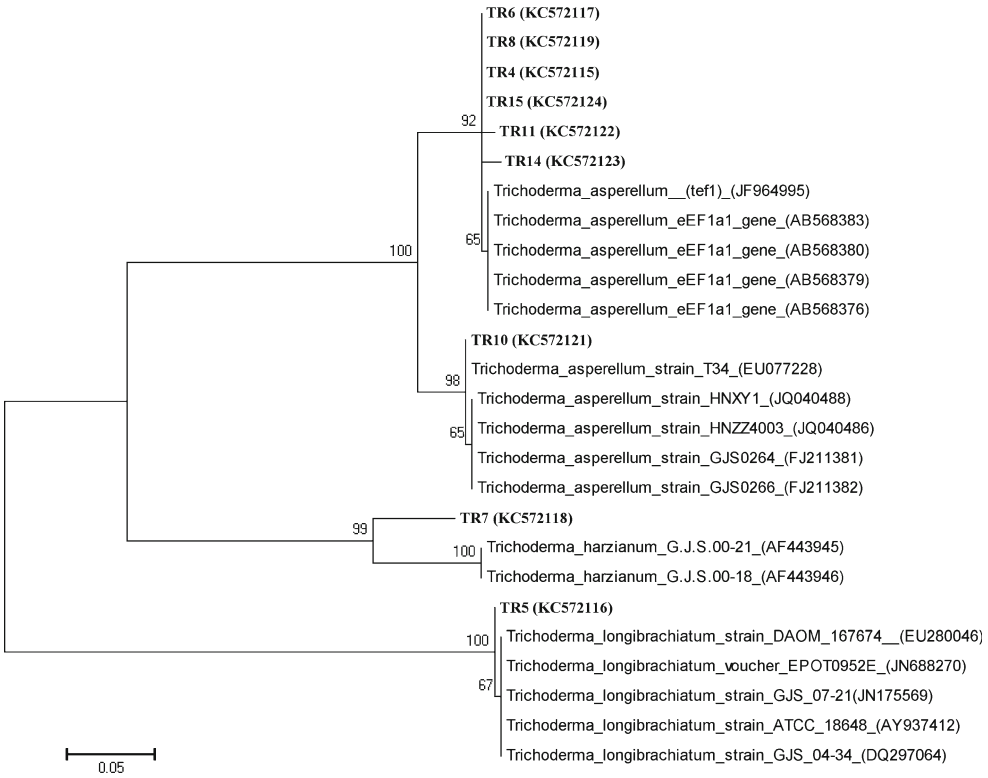

Fig. 2 A neighbour-joining phylogenetic tree showing relationships among selected *Trichoderma* strains using *tef1* gene sequences. The study was performed using MEGA software version 5. Reference sequences included in the analysis were obtained from the NCBI Genbank database based on BLAST analysis. The numbers of nodes represents bootstrap values (2,000 replicates)

evaluated. The highest number of amplification products (79) was obtained with the primer pair E+AT/T+AA, while the lowest (45) was with the E+GT/T+TC pair; the average number of bands among a total of eight primer pairs was 67.6. The number of polymorphic fragments detected by each primer varied from 45 to 77, with an average of 62.8. The highest number of polymorphic bands (77) was produced by the primer pair E+AA/T+AT whereas the primer E+GT/T+TC generated the lowest number of polymorphic bands (45) (Table 3).

The dendrogram constructed based on the UPGMA cluster analysis displayed considerable variation among the isolates. The isolates were grouped according to their species with *T. asperellum* isolates forming a single cluster, and *T. harzianum* forming an out group (Fig. 3). The robustness of the clustering was well supported by high bootstrap values. The pattern of clustering of isolates was not associated with their geographical origin or degree of antagonism.

The cophenetic correlation coefficient between dendrogram and the original similarity matrix were significant for AFLP (r=0.825).

Discussion

Biocontrol of diseases and pests of crops using microbial antagonists seems to be a reliable alternative to chemical fungicides, which is eco-friendly, safe and a potential component of integrated disease management (IDM) (Moenne-Loccoz et al. 2001). Biocontrol agents (BCAs) have specific advantages over synthetic fungicides, including fewer non-target and environmental effects, efficacy against fungicide-resistant pathogens, and reduced probability of resistance development in pathogens (Vinale et al. 2008). *Trichoderma* spp. are common saprophytic fungi in the rhizosphere, which have received considerable attention as a potential BCA. The primary objective of this study was to isolate and characterize *Trichoderma* spp. antagonistic to pathogen *P. colocasiae*. Our results indicate that *Trichoderma* spp. is highly efficient in restricting the growth of the pathogen.

The genus *Trichoderma* comprises a great number of strains acting as BCAs through the activation of direct or indirect mechanisms (Harman 2004; Vinale et al. 2008). Direct effects include competition for nutrients or space, production of lytic enzymes, inactivation of the pathogen's enzymes and parasitism. Indirect effects include all those aspects that produce morphological and biochemical changes in the host plant including induced resistance (Harman 2006). They have been reported to produce a plethora of secondary metabolites possessing antimicrobial activity (Sivasithamparam and Ghisalberti 1998; Vinale et al. 2008). The in vitro confrontation assay has shown to be a useful and reliable method for identifying the biocontrol efficacy of *Trichoderma* strains (Hermosa et al. 2000). In the present study, the most efficient strain overall was TR7, which completely inhibited *P. colocasiae* growth in both dual culture and diffusible

Table 3 Summary statistics for *Trichoderma* isolates from different regions of India based on AFLP amplification

AFLP Marker	Primer	Sequence (5′-3′)	No. of bands scored	No. of polymorphic bands	Mean no. of bands	Polymorphism (%)
1	E+AT/T+AA	CTC GTA GAC TGC GTA CC AT/TACT CAGGACTGGCAA	79	68	44	86
2	E+AA/T+AT	CTC GTA GAC TGC GTA CC AA/TACT CAGGACTGGC AT	78	77	33.5	98.7
3	E+AG/T+AG	CTC GTA GAC TGC GTA CC AG/TACT CAGGACTGGC AG	54	51	23.3	94.4
4	E+AG/T+TG	CTC GTA GAC TGC GTA CC AG/TACT CAGGACTGGCTG	74	72	38.2	97.2
5	E+AC/T+GG	CTC GTA GAC TGC GTA CC AC/TACT CAGGACTGGCGG	71	64	37.6	90.1
6	E+GT/T+TC	CTC GTA GAC TGC GTA CC GT/TACT CAGGACTGGCTC	45	45	26	100
	Total		406	377	202.6	
	Average		67.6	62.8	33.7	94.4

assays. The remaining strains displayed a range of antagonist activity from 40% to 100 %. It should be noted that *Trichoderma* isolates belonging to the same species (in our case *Trichoderma asperellum*) showed differential antagonist activity against *P. colocasiae*, which probably reflects their genetic makeup. This discrepancy in antagonistic activity among strains of the same species has been previously observed (Anees et al. 2010). From the current results, it can be commented that antagonism is not a property of the species but depends on the genetic composition of the individual strains, as different strains of the same species can exhibit varying potentials of biocontrol. The strains which can express rapidly and efficiently their genes involved in antagonistic activities in the presence of host are in fact better antagonists (Scherm et al. 2009).

BCAs are known to produce cell wall–degrading enzymes, such as chitinases, β-1,3-glucanases, cellulases and proteases which are involved in the antagonistic activity against phytopathogenic fungi (Harman et al. 2004). It is known that *Trichoderma* species secretes hydrolytic enzymes (especially chitinases and β-1,3-glucanases) and detects the presence of another fungus by sensing the molecules released from the host by enzymatic degradation (Harman et al. 2006). In the present study, selected *Trichoderma* isolates were analyzed for their ability to secrete extracellular enzymes viz. chitinase (EC 3.2.1.14) and β -1,3 glucanase (EC 3.2.1.3.9). The results showed that there was a positive relationship between the activity of these enzymes and the degree of antagonism measured in the in vitro antagonistic assays. The activity of these enzymes varied with different isolates of *Trichoderma* species. As the cell walls of pathogenic fungi consist predominantly of chitin, glucan and proteins, a successful antagonist should have all or a part of these enzymes, which play a significant role in the cell wall lysis of the pathogen (Vinale et al. 2008). In our case, as the cell walls of oomycetes consist predominantly of (1–3) -β -D-glucans, (1–6) -β-D-glucans, and cellulose (Bartnicki-García 1968), we speculate that the glucanases secreted by the *Trichoderma* strains play a predominant role in restricting the growth of *P. colocasiae* as observed earlier by Haran et al. (1996). Nevertheless, it is probable that the biological control activity cannot be ascertained to a single enzyme; rather a combination of secreted lytic enzymes is necessary for optimum activity (Zaldívar et al. 2001).

One *Trichoderma* strain (*T. harzianum*, TR7) due to its high antagonistic potential in vitro was selected for its biocontrol activity in vivo using a detached leaf assay. The results revealed the strain is not only biologically active in vitro but also equally effective in vivo in controlling leaf blight disease. It is therefore a potentially important biological control agent.

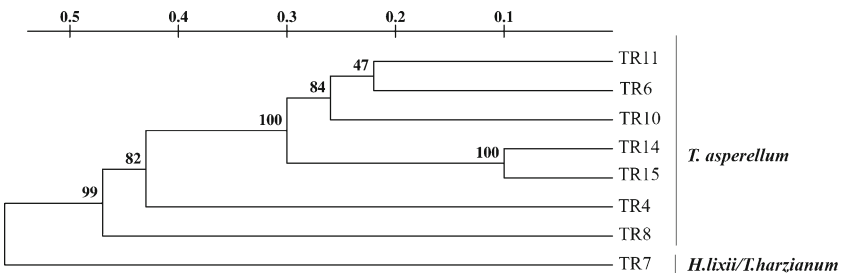

Fig. 3 UPGMA dendrogram depicting genetic relationships among selected *Trichoderma* strains based on AFLP analysis. The numbers of nodes represents bootstrap values (2,000 replicates)

The identification of *Trichoderma* species has been primarly based on morphology. However, the few morphological characters with limited variation may lead to an overlap and misidentification of the strains (Kullnig et al. 2001). Thus, the morphological characteristics need to be combined with molecular data obtained from DNA sequencing (Samuels 2006). Generally, a single gene is not considered sufficient for molecular identification as some closely related species share the same sequences of a target gene (for example ITS region, Samuels 2006). In the present study, we have used a multigene approach (ITS and *tef1* region) for accurately identifying the *Trichoderma* strains to the species level. Among the *Trichoderma* strains identified in this study, the most abundant species was *T. asperellum. Trichoderma* spp. are recognized as cosmopolitan soil fungi, colonizing a wide range of habitats and ecological niches (Schuster and Schmoll 2010). In addition, the isolation of *Trichoderma* species has been explained by environmental parameters such as soil temperature, moisture, pH, organic matter and nutrient content present in the sampling site (Carreiro and Koske 1992; Klein and Eveleigh 1998). In the present study, the *Trichoderma* isolates were obtained from different origins of India, indicating that *T. asperellum* is widely distributed. Only one isolate belonged to the species *T. harzianum.* Until now, few data are available concerning the biodiversity of *Trichoderma* in soil. Nevertheless, previous studies have rather reported *T. harzianum* as the dominant species among soil isolates (Zhang et al. 2005; Migheli et al. 2009). In our study, the number of isolates is too small to draw a definite conclusion in terms of *Trichoderma* species diversity.

The UPGMA dendrogram based on the AFLP analysis revealed a high degree of genetic diversity among the isolates. Our results were in agreement with the study of Goes et al. 2002, who also found high variability in the *Trichoderma* strains of the same species using RAPD markers. Isolates from the same region failed to cluster together suggesting their divergence. No correlation in grouping could be assessed from the dendrogram in terms of an antagonistic property or extracellular enzyme production. The diversity observed among the isolates may be the outcome of sexual recombination as it is becoming increasingly clear from the recent studies that many of the *Trichoderma* spp. thought to be asexual display an evolutionary history of sexual recombination (Druzhinina et al. 2011). The variability present among the isolates may be further elucidated by the fact that the isolates used for the study were obtained from diverse geographical origins and it is possible that they have developed specific properties to adapt to the different environmental conditions. However, more number of isolates representing diverse geographical origins has to be analyzed to ascertain the role of environmental factors in generating genetic diversity.

In conclusion, a polyphasic approach allowed us to identify the *Trichoderma* isolate (TR7) which exhibited the highest antagonist activity (both in vitro and in vivo) against the phytopathogen *P. colocasiae.* The identified isolate could be used as a promising candidate for the biological control of taro leaf blight disease. The knowledge generated through this study on genotypic and phenotypic diversities of *Trichoderma* strains will serve as a reference source for future studies concerning biological control of *P. colocasiae.* Although the study resulted in the identification of the best antagonistic strains of *Trichoderma*, it should be noted that the effective strain has yet to be evaluated in the field. Biocontrol field trials are underway and the results will be communicated shortly.

Acknowledgments The funding provided for conducting the research work by the Indian Council of Agricultural Research, New Delhi, under IISR outreach programme is gratefully acknowledged. Thanks are due to Mr. Ragesh, Ph.D scholar, CTCRI for his assistance in statistical analysis. The authors wish to thank the editor and the reviewer for their valuable comments in improving the manuscript.

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