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Specific RT-PCR Assays for the Detection of *Trichoderma harzianum (Th azad)* in Rhizopsperic Soil Sample of Uttar Pradesh India

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Strain identification *in situ* is an important factor in the monitoring of microorganisms used in the field. In this study, we demonstrated the use of sequence-characterized amplified region (SCAR) markers to detect genomic DNA from *Trichoderma harzianum* Th azad from soil. Two primers (SCAR A1/SCAR A1c) were tested against DNA of 49 isolates of *Trichoderma* spp. and amplified a 900-bp fragment from *T. atroviride* 11 and a 1.5-kb fragment from *T. harzianum* Th azad, using an annealing temperature of 68°C. These fragments showed no significant homology to any sequence deposited in the databases. The primer pair, BR1 and BR2, was designed to the 1.5-kb fragment amplified from *T. harzianum* Th azad, generating a SCAR marker. To test the specificity of these primers, experiments were conducted using the DNA from 49 *Trichoderma* spp. strains and 22 field soil samples obtained from different agro-geographical condition of UP. PCR results showed that BR1 and BR2 amplified an 830-bp fragment unique to *T. harzianum* Th azad. Assays in which total DNA was extracted from sterile and nonsterile soil samples, inoculated with spore or mycelium combinations of *Trichoderma* spp. strains, indicated that the BR1 and BR2 primers could specifically detect *T. harzianum* Th azad in a pool of mixed DNA. No other soil-microorganisms containing these sequences were amplified using these primers. To test whether the 830-bp SCAR marker of *T. harzianum* Th azad could be used in real-time PCR experiments, new primers (CSA-Th azadf and CSA-Th azad) conjugated with a TaqMan fluorogenic probe were designed. Real- time PCR assays were applied using DNA from sterile and nonsterile soil samples inoculated with a known quantity of spores of *Trichoderma* spp. strains.

Keywords: RT-PCR, *Trichoderma*, Genomic DNA, SCAR marker.

Species of the fungus *Trichoderma*, a genus of *Hyphomycetes*, are ubiquitous in the environment, but especially in soil. They have been used in a wide range of commercial applications including the production of enzymes [De la Cruz, J, *et al*. (1999), Kubicek, CP *et al*. (1998), Lorito, M *et al*. (1998)] and in the biological control of plant diseases [Hjeljord, L, *et al*. (1998), Samuels, GJ (1996)]. Characterization and identification of strains at the species level is the first step in utilizing the full potential of fungi in specific applications [Lieckfeldt, E, *et al*. (1999)]. Biological assays, carried out in the laboratory, are often an effective and rapid method for identifying strains with biocontrol activity [Grondona I *et al*. (1992)].To predictably and successfully use bio-control agents to combat plant disease in the field,

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it is essential that their biology and ecology be well under- stood [Lo, CT *et al*. (1996)]. In addition, the release of bio control agents into the environment has created a demand for the development of methods to monitor their presence or absence in soil. Therefore, monitoring population dynamics in soil is of much importance. Previous methods employed to identify strains of *Trichoderma* spp. in soil samples have included the use of dilution plates on selective media. However, this method does not distinguish between indigenous strains and artificially introduced ones [Kimura, H *et al*. (1999)]. Staining techniques, based on reporter genes such as the b-glucuronidase gene (GUS), have been used to study host–pathogen interactions *in vivo* [Green, H, *et al*. (1995)] and monitoring commercial *Trichoderma* strains [Freeman, S *et al*. (2002), Lo, CT *et al*. (1998)]. This method is not suitable for detecting or monitoring *Trichoderma* strains under natural conditions since this would involve the release of genetically modified organisms into the environment.

In recent years, several PCR-based molecular techniques have been used to detect and discriminate among microorganisms. Sequence analysis of the internal transcribed spacer region 1 (ITS1) of the ribosomal DNA has been helpful in the neotypification, description, and characterization of species in the genus *Trichoderma* [Gams, W, *et al*. (1998), Shahid M *et al*. (2013a, 2014f), Grondona, I *et al*. (1997), Hermosa, MR *et al*. (2000), Lieckfeldt, E, *et al*. (1999), Muthumeenakshi, S. *et al*. (1994), Ospina-Giraldo, MD *et al*. (1999)]. In addition, the analysis of DNA sequences from multiple genetic loci has been used to establish the phylogenetic relationship of the species within *Trichoderma* [Chaverri, P. *et al*. (2003), Kullnig-Gradinger, C. *et al*. (2002)]. Another useful method for the identification of *Trichoderma* strains is the randomly amplified polymorphic DNA (RAPD) technique [Zimand, G *et al*. (1984), Shahid M *et al*. (2013b, 2014g)]. In a previous study, we identified RAPD fragments that were able to discriminate between different biocontrol agents of *Trichoderma* spp. [Hermosa, MR *et al*. (2001)]. By converting a RAPD- derived fragment, amplified from strain *T. atroviride* 11, into a sequence-characterized amplified region (SCAR) we showed that this marker was specific to *T. atroviride* 11. The generation of a SCAR marker for this strain is of particular interest because *T. atroviride* 11 has been shown to be an efficient biocontrol agent against phy- topathogenic fungi such as *Phomabetae*, *Rhizoctonia so- lani*, *Sclerotinia sclerotiorum*, and *Polymyxa betae*, the vector of the sugar beet rhizomania disease virus [Campos, T *et al*. (2001), Grondona, I, *et al*. (2001), Grondona, I. *et al*. (1992)].

Although conventional PCR has become an attractive tool for the detection of specific microorganisms in microbial systems, this technique does not allow accurate quantification of DNA because of the variability in the efficiency of amplification between PCR reactions [Raeymaekers, L (1998)]. This limiting factor has been overcome by the emergence of new techniques, capable of quantifying nucleic acids in vitro. Real-time PCR is based on the use of TaqMan probes or SYBR Green I dyes. Both systems measure the intensity of a fluorescent signal proportional to the quantity of DNA generated during the PCR amplifica- tion. Quantitative real-time PCR is widely used in medicine for the diagnosis of clinical viruses [Kimura, H. *et al*. (1999).], bacteria [Hein, I, *et al*. (2001), Ke, D, *et al*. (2000)], protozoa [Lin *et al*. (2000)], and fungi [Loeffler, J. *et al*. (2000)]. Although it is used in plant pathology studies [Schena, L. *et al*. (2002)], there are few references regarding its application to quantifying fungi from soil [Filion, M. *et al*. (2002)].

The aim of the present study was to develop specific PCR-based markers to detect *T. harzianum Th azad* in soil. Also, two primers, QTh azadf and QTh azadr, designed from the 837-bp SCAR marker, were used to quantify fungal DNA of this strain extracted from soil samples using real-time PCR.

METHODS

Fungal Strains, Growth Conditions, and DNA Extraction

The 49 *Trichoderma* spp. were used in this study are shown in Table 1. Each strain was cultured on Potato Dextrose Agar medium (PDA, Himedia) and stored at -80°C in 10% glycerol. Petri dishes containing PDA medium were inoculated

with a 0.5-cm diameter agar plug, cut from the growing edge of a 4-day colony for each strain, and grown in the dark at 25 C. Spores were harvested after 6 or 7 days, by adding 2 mL of water to the plates and scraping the culture with a rubber spatula. The suspensions were filtered through a double layer of cheese- cloth to separate large mycelia fragments from conidia. Spore concentrations were adjusted to 1 x 10^9 conidia mL). Mycelia, for each strain, were obtained by inoculating flasks containing 100 mL of Potato Dextrose Broth (PDB, Himedia Laboratories) and growing the cultures at 25°C for 48h on orbital shaker (120 rpm). Mycelia were harvested and washed with sterile distilled water, frozen, and lyophilized. Total DNA was extracted from 50 mg of freeze-dried mycelia [Shahid M. *et al*. 2014a, Mondéjar R L et al (2010), Raeder, U. *et al*. (1985)].

DNA extracted from 250 mg of soil was obtained using the Fast DNA SPIN (for soil) kit (according to manufacturer's instructions, with a modification in the lysis step. Lysis was carried out using the solution provided in the kit. However, the samples were incubated at 65 C and vortexed every 5 min for 30 min. The DNA extracted from each sample was resuspended in 100 lL of DES buffer accompanying the kit.

Screening *Trichoderma* Strains for SCAR Markers

SCAR A1 (5-GGAAGCTTGGCGTTTATTGTA CAAAG-3) and SCAR A1c (5-GGAAGCTTGGGTATT GAGCTGGGCCT-3) primers, designed from a previous study involving *T. atroviride* 11 [Hermosa, MR. *et al*. (2001)], were used to test the amplification of DNA from 50 strains of *Trichoderma* spp. Each PCR reaction contained 50 ng of fungal DNA (Table 1), 2.5 U of DNA Taq polymerase, 1· DNA polymerase buffer , 0.2 mM of each deoxynucleoside triphosphate, 1.5 mM of MgCl2, and 0.32 lM of each primer in a total volume of 50 lL. PCR conditions included an initial denaturation of 5 min at 94^0 C, followed by 35 cycles of 1 min at 94^0 C, 1 min 30 s at 68 C, and 2 min at 72^0 C, with a final extension step of 72^0 C for 7 min, using a DNA PTC-100 thermal cycler. PCR products were analysed by electrophoresis in a 1.2% agarose gel in 1· TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA [pH 8]), stained with ethidium bromide. The molecular size marker was / X174- HaeIII (Promega, Madison, WI, **USA**).

Generation of a SCAR Marker for *T. harzianum* Th azad

A 1.5-kb PCR fragment was excised from an agarose gel and the DNA was purified with the GENE CLEAN kit according to the manufacture's specifications. The fragment was cloned into the pGEM-T Easy vector (Promega) with T4 DNA Ligase [Vieira PM et al (2013), Sambrook, J. *et al*. (1989)]. Plasmid DNA was extracted using a mini-plasmid preparation protocol [Hanahan, D (1985)]. The 1.5-kb fragment was sequenced using fluorescent terminators on an AB RT PCR.

Their identification is important in developing a potential strain for further analysis. These isolate belongs to 8 different species of *Trichoderma* genus viz. *Trichoderma viride* (30 strains), *Trichoderma harzianum, Trichoderma asperellum, Trichoderma longibrachiatum* (7 strains), *Trichoderma atroviride* (8 strains), *Trichoderma koningii, Trichoderma virens and Trichoderma reesei* collected from rhizospheric soil of chickpea, pigeonpea and lentil crop. They are finally deposited to culture bank NBAIM, Mau and allotted with a unique NBAIM Accession number.

Real-Time PCR Assays. One primer set, QTh azadf (5-TGGCGTTGAATTGAGTTTGTGT-3) and QTh azadr (5-CCCTCCGTA TGGGTTT TAAGGT-3) , was designed using the Primer Express software program version 2.0.0 (Applied Biosystems). These oligonucleotides, designed from the 837-bp SCAR marker, flanked a 72-bp DNA fragment specific for identifying the strain *T. harzianum Th* azad. The specificity of this primer set was tested against DNA from all 50 *Trichoderma* spp. strains, using a conventional PCR machine, and on annealing temperature of 59°C for the reactions.

The QTh azadr and QTh azadf primers were used in real time-PCR experiments with 6-carboxyfluorescein (6-FAM) as the reporter fluorochrome & 6-carboxyte- tramethylrhodamine (TAMRA) as the quencher. The 20- lL master mixture contained 2 lL of undiluted DNA template from different sources, 10 lL of TaqMan Universal PCR Master Mix (Applied Biosystems), a 0.45-lM concentration of each primer, and 0.5 lM of a TaqMan fluorescent DNA probe (Applied Biosystems). Thermal cycler conditions were as

follows: 50 C for 2 min and95 C for 10 min, and 40 cycles of PCR amplification at 95 C for 15 s and 59 C for 30 s.

A standard curve, based on threshold cycles (Ct), was created using plasmid DNA containing the 837-bp SCAR marker, and the QTh azadf/QTh azadr primer pair. Plasmid DNA amounts, quantified by spectrophotometry and serially diluted, were 200 pg, 20 pg, 2 pg, 200 fg, 20 fg, and 2 fg. Sterile water was used as a negative control to replace template DNA in PCR reactions (NTC). Ct values were calculated by the ABI Prism 7000 SDS software program (Applied Biosystems) to indicate significant fluorescence signals rising above background during the early cycles of the exponentially growing phase of the PCR amplification process. The standard curve was obtained by plotting the Ct value, defined by the crossing cycle number, versus the logarithm of the quantity of the different plasmid DNA samples. Two microlitre of undiluted DNA from the A, B, C was included as template in the real-time PCR reactions Two microlitre of genomic DNA was also included as positive control. The amount of template DNA was calculated by interpolating the cycle threshold with the standard curve, determined by the ABI Prism 7000 SDS software program, and by the application of a correction factor. DNA quantities were corrected by dividing by 35, a figure that represented the ratio between the 110-bp target fragment and the total size of the plasmid DNA used to generate the standard curve. All reactions were carried out in triplicate.

RESULTS

SCAR Marker Generation for *T. harzianum* Th azad

Two PCR products were amplified using SCAR A1 and SCAR A1c primers: one 990-bp fragment corresponding to strain *T. atroviride* 11 and one 1.5-kb fragment corre- sponding to *T. harzianum* Th azad . The 990-bp fragment was shown to be diagnostic of *T. atroviride* in work previously done using 17 strains of *Trichoderma* [Hermosa, MR. *et al*. (2001)]. Two primers, BR1 and BR2, were designed to the 1.5-kb fragment from *T. harzianum* Th azad and used to amplify DNA extracted from 49 strains of *Trichoderma* spp..Of these samples, only one 1.5 kb amplicon, specific to a sample containing only *T. harzianum* Th azad DNA, was amplified using an annealing temperature of 65°C. No PCR products were detected in any of the other 48 PCR reactions.

Detecting *Trichoderma* in Inoculated Soils

Sample A, a sterile soil artificially inoculated with a mix of 49 *Trichoderma* spp. strains at a concentration of 109 spores per 10 g of soil, yielded 1.6 to 5.6 lg of DNA per g (w/w) of soil. No differences in the amount of DNA were observed in soil samples. The amount of DNA extracted from soil sample, a sterile soil inoculated only with *T. harzianum* Th azad at a concentration of 109 spores per 10 g of soil, was not detected in agarose gel.

BR1 and BR2 primers were used to screen the DNA from the soil samples (Table 1) to discriminate between samples with or without *T. harzianum* Th azad. The diagnostic *1.5* Kb PCR fragment was amplified in soil samples which included spores of *T. harzianum* Th azad. No PCR products were amplified in samples, where spores of *T. harzianum* Th azad were not added (Fig. 1). Identical results were observed when mycelia instead of spores of the *Trichoderma* spp. strains were used to inoculate the soils. The quantities of DNA extracted from soils inoculated with mycelia were 60% higher than the amount extracted using spores as the inoculant.

Screening and Quantification of Fungal DNA Using Real-Time PCR

The QTh azadf/QTh azadr primer pair was used in real-time PCR. A standard curve was created using 4-fold dilutions series of the plasmid containing the 1.5kb SCAR fragment and both primers. The threshold was set to a value of 0.50 with a baseline ranging from cycle 1 to 36. The standard curve showed a linear correlation between input DNA and cycle threshold (**Fig. 2**), with a correlation coefficient (r2) of 0.100754. Significant fluorescent signals were observed for reactions containing DNA extracted from sample , inoculated with 109 spores of *T. harzianum* Th azad per 10 g of soil (Table 1). Genomic DNA of *T. harzianum* Th azad, included as positive control, was also detected. No significant fluorescent signals, rising above background, were detected for sample, which did not contain *T. harzianum* Th azad strain. In addition, no fluorescence was observed for sample , a nonsterile and artificially inoculated

Table 1. *Trichoderma* isolates used in this study

S. No.	Strain code	Source	ITCC No.	Fungus Identified	Crop	GPS Location
1	6 CP	Sultanpur	7442/09	*T.atroviride*	Chickpea	Latitude: 26.2500 ºN Longitude: 79.0000ºE
2	24CP	Sitapur	7443/09	*T.atroviride*	Chickpea	Latitude: 27.5700ºN Longitude: 80.6800ºE
3	71 L	Hardoi	7445/09	*T.atroviride*	*Lentil*	Latitude: 26Ú 292 28.3232 2 Longitude: 80Ú 182 26.3612 2
4	115 L	Bahraich	7446/09	*T.atroviride*	*Lentil*	Latitude: 27.7500ºN Longitude: 81.7500ºE
5	52 L	Unnao	7447/09	*T.atroviride*	*Lentil*	Latitude: 26.5500 ºN Longitude: 80.4800ºE
6	105 CP	Etawah	7451/09	*T.atroviride*	Chickpea	Latitude: 26.7700 ºN Longitude: 79.0300ºE
7	75 PP	Auriya	7448/09	*T.atroviride*	Pigeon pea	Latitude: 26.4700 ºN Longitude: 79.5200ºE
8	126 PP	Kanpur Dehat	7449/09	*T.atroviride*	Pigeon pea	Latitude: 26.2277 Longitude: 79.8370
9	21 PP	Kaushambi	7437/09	*T.longibrachiatum*	Pigeon pea	Latitude: 26Ú 342 27.612 2 Longitude: 79Ú 182 24.6232 2
10	31PP	Allahabad	7438/09	*T.longibrachiatum*	Pigeon pea	Latitude: 25.4358 Longitude: 81.8463
11	81PP	Mirzapur	7439/09	*T.longibrachiatum*	Pigeon pea	Latitude: 25.1500 ºN Longitude: 82.6000ºE
12	100 PP	Sonbhadra	7440/09	*T.longibrachiatum*	Pigeon pea	Latitude: 24.6897 ºN Longitude: 83.0653ºE
13	120 PP	Bhadoi	7441/09	*T.longibrachiatum*	Pigeon pea	Latitude: 25.3932 Longitude: 82.5657
14	28 CP	Barabanki	7444/09	*T.longibrachiatum*	Chickpea	Latitude: 26.9200 ºN Longitude: 81.2000ºE
15	5 CP	Kanpur Nagar	7450/09	*T.longibrachiatum*	Chickpea	Latitude: 25Ú 82 34.8212 2 Longitude: 81Ú 592 2.9792 2
16	8 CP	Kanpur Nagar	8305/11	*T. viride*	Chickpea	Latitude: 26.4600 ºN Longitude: 80.3300ºE
17	11 CP	Allahabad	8306/11	*T. viride*	Chickpea	Latitude: 25.4358 Longitude: 81.8463
18	17 CP	Kaushambi	8307/11	*T. viride*	Chickpea	Latitude: 26Ú 342 27.612 2 Longitude: 79Ú 182 24.6232 2
19	33 CP	Sitapur	8308/11	*T. viride*	Chickpea	Latitude: 27.5700ºN Longitude: 80.6800ºE
20	34 CP	Hardoi	8309/11	*T. viride*	Chickpea	Latitude: 26Ú 292 28.3232 2 Longitude: 80Ú 182 26.3612 2
21	35 CP	Fatehpur	8310/11	*T. viride*	Chickpea	Latitude: 25.9300 ºN Longitude: 80.8000ºE
22	66 CP	Auriya	8311/11	*T. viride*	Chickpea	Latitude: 26.4700 ºN Longitude: 79.5200ºE
23	89 CP	Unnao	8312/11	*T. viride*	Chickpea	Latitude: 26.5500 ºN Longitude: 80.4800ºE
24	102 CP	Etawah	8313/11	*T. viride*	Chickpea	Latitude: 26.7700 ºN Longitude: 79.0300ºE
25	119 CP	Kanpur Dehat	8314/11	*T. viride*	Chickpea	Latitude: 26.2277

						Longitude: 79.8370
26	01 PP	Hardoi	8315/11	*T. viride*	*Pigeonpea*	Latitude: 26Ú 292 28.3232 2 Longitude: 80Ú 182 26.3612 2
27	09 PP	Kanpur Nagar	8316/11	*T. viride*	*Pigeonpea*	Latitude: 25Ú 82 34.8212 2 Longitude: 81Ú 592 2.9792 2
28	14 PP	Barabanki	8317/11	*T. viride*	*Pigeonpea*	Latitude: 26.9200 ºN Longitude: 81.2000ºE
29	17 PP	Fatehpur	8318/11	*T. viride*	*Pigeonpea*	Latitude: 25.9300 ºN Longitude: 80.8000ºE
30	29 PP	Unnao	8319/11	*T. viride*	*Pigeonpea*	Latitude: 26.5500 ºN Longitude: 80.4800ºE
31	42 PP	Bahraich	8320/11	*T. viride*	*Pigeonpea*	Latitude: 27.7500ºN Longitude: 81.7500ºE
32	67 PP	Auriya	8321/11	*T. viride*	*Pigeonpea*	Latitude: 26.4700 ºN Longitude: 79.5200ºE
33	74 PP	Sitapur	8322/11	*T. viride*	*Pigeonpea*	Latitude: 27.5700ºN Longitude: 80.6800ºE
34	78 PP	Etawah	8323/11	*T. viride*	*Pigeonpea*	Latitude: 26.7700 ºN Longitude: 79.0300ºE
35	124 PP	Kanpur Dehat	8324/11	*T. viride*	*Pigeonpea*	Latitude: 26.2277 Longitude: 79.8370
36	13 L	Kanpur Nagar	8325/11	*T. viride*	*Lentil*	Latitude: 26.4600 ºN Longitude: 80.3300ºE
37	104 L	Etawah	8331/11	*T. viride*	*Lentil*	Latitude: 26.7700 ºN Longitude: 79.0300ºE
38	35 L	Sitapur	8326/11	*T. viride*	*Lentil*	Latitude: 27.5700ºN Longitude: 80.6800ºE
39	68 L	Hardoi	8327/11	*T. viride*	*Lentil*	Latitude: 26Ú 292 28.3232 2 Longitude: 80Ú 182 26.3612 2
40	74 L	Auriya	8328/11	*T. viride*	*Lentil*	Latitude: 26.4700 ºN Longitude: 79.5200ºE
41	89 L	Gonda	8329/11	*T. viride*	*Lentil*	Latitude: 27.2500 ºN Longitude: 82.0000ºE
42	100 L	Faizabad	8330/11	*T. viride*	*Lentil*	Latitude: 26.7800 ºN Longitude: 82.1300ºE
43	109 L	Allahabad	8332/11	*T. viride*	*Lentil*	Latitude: 25.4358 Longitude: 81.8463
44	117 L	Sultanpur	8333/11	*T. viride*	*Lentil*	Latitude: 26.2500 ºN Longitude: 79.0000ºE
45	119 L	Kanpur Dehat	8334/11	*T. viride*	*Lentil*	Latitude: 26.2277 Longitude: 79.8370
46	*Th azad*	CSA Univ. Farm	6796/12	*T.harzianum*	Chickpea	Latitude: 26.4912 ºN Longitude: 80.3070ºE
47	*T.spe* /(CSAU)	CSA Univ. Farm	8940/12	*T.asperellum*	*Pigeonpea*	Latitude: 26.4912 ºN Longitude: 80.3070ºE
48	*T.kon* /(CSAU)	CSA Univ. Farm	5201/13	*T.koningii*	*Pigeonpea*	Latitude: 26.4912 ºN Longitude: 80.3070ºE
49	*T.vire* /(CSAU)	CSA Univ. Farm	4177/13	*T. virens*	*Pigeonpea*	Latitude: 26.4912 ºN Longitude: 80.3070ºE

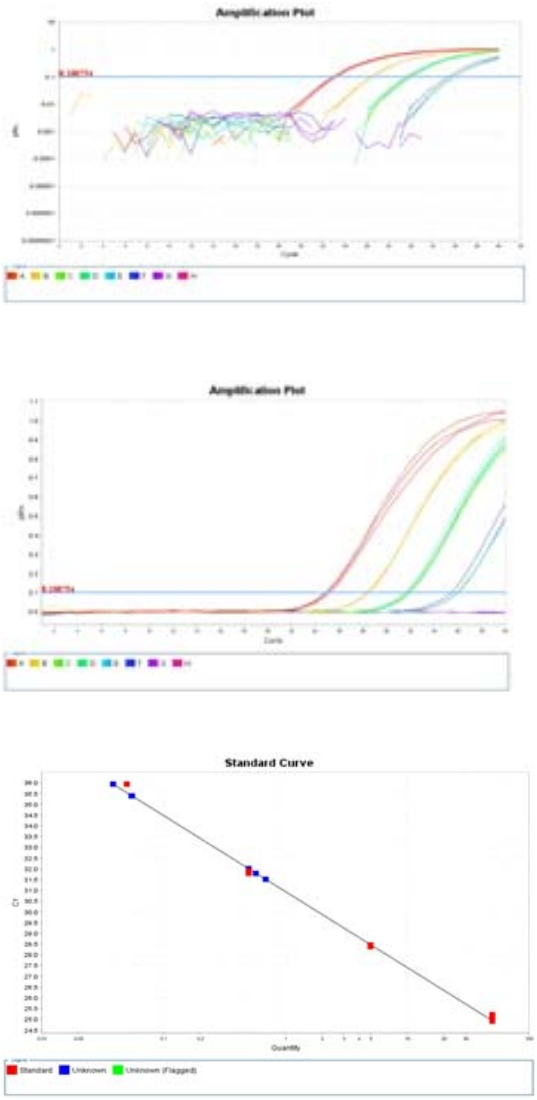

Fig 1. Showing expression of *T. harzianum* Th azad DNA

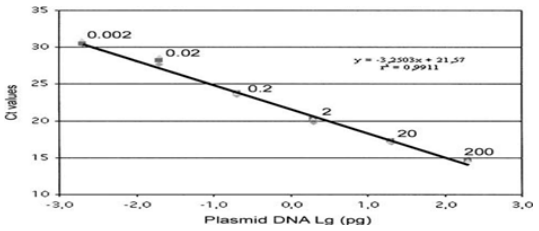

Fig. 2. Standard curve with the correlation coefficient (r^2) ob- tained by plotting the cycle threshold (Ct) against the input DNA plasmid quantity (logarithm scale) after real-time PCR

soil, which included 49 *Trichoderma* spp. strains distinct from *T. harzianum* Th azad. Thus, indicating that primers QTh azadf and QTh azadr did not amplify the DNA from other microorganisms or *Trichoderma* spp. strains present in this soil. The absence of *T. harzianum* Th azad propagules in sample was confirmed by plating triplicates of 100 mg of soil on *Trichoderma* specific medium, TSM [Askew, DH. *et al*. (1993)]. For sample A, fluorescent signals were detected, but this result was not consistent within the three replicates done for each experiment and could not be considered as a real quantification.

Strains identification *in situ* is an important factor in the monitoring of microorganisms used in the field. In this study, we demonstrated the use of Sequence Characterized Amplified Region (SCAR) markers to detect genomic DNA from *Trichoderma* strains from soil. Two primers (SCAR A1 (5 ' -GGAAGCTTGG CGTTTATTGTACAAAG-3 ') and SCAR A2 (5 ' -GGAAGCTTGGGTATTGAGCTGGGCCT-3') were tested against DNA of 49 isolates of *Trichoderma* spp. and a 1.5 kb fragment from *T. harzianum* Th azad, using annealing temperature of 68ºC. These fragments showed no significant homology to any sequence deposited in the database.

DISCUSSION

The identification of organisms on the basis of DNA investigation requires the characterization of discrimi- nating DNA targets. In fact, this is especially important in the case of *Trichoderma* strains widely used in the bio- control of soil-borne plant-pathogens. Although *T. harzianum* is the species most frequently used in biological control, a search of the sequences from commercial strains revealed that *T. asperellum*, *T. atroviride* and *T. koningii* were also common species used [Hermosa, MR. *et al.* (2000), Samuels, GJ (1996)]. Moreover, because of the difficulty of monitoring the biocontrol activity of a given strain in natural environments, the development of molecular characters is needed. A recent work describes the cotransformation of a *T. harzianum* strain with green fluorescent protein and GUS genes [Bae, YS. *et al*. (2000)] to study its ability to colonize soil and sclerotia of a plant pathogenic fungus. These genes provide a

valuable tool for the detection and monitoring of specific strains of *T. harzianum* released into the soil. However, the practical use of these transformants is difficult because of the public concern about genetic modified organisms in natural environments.

PCR has provided a reliable method for the identification and detection of microorganisms. Specific primers have been designed from ITS regions or rRNA genes of fungi such as *Rosellinia necatrix* [Schena, L. *et al*. (2002)] or *Glomus intraradices* [Filion, M. *et al*. (2003)], and specific primers from the *Fusarium solani f. sp. phaseoli* translation elongation factor 1 alpha (tef1) gene [Filion, M. *et al*. (2003)] have permitted the identification of these fungi. Several studies have indicated that some *Trichoderma* species are closely related [Gams, W, *et al*. (1998), Kullnig-Gradinger *et al*. (2002)]. Divergence values of ITS1 and ITS2 regions, ranging from 1.2 to 6.5%, 0 to 5.2% and 1.9 to 14.6%, have been shown for three sections of this genus: *Longibrachiatum*, *Trichoderma*, and *Pachybasium* [Shahid M. *et al*. 2013c, 2014b, Kuhls, K. *et al*. (1997)]. Similarly, sequence analysis of ITS1 revealed *T. atroviride*, *T. asperellum*, and *T. harzianum* differ by only 0 to 3% [Hermosa, MR. *et al*. (2000)]. A DNA sequence data analysis of four genes (ITS regions, tef1 a, calmodulin, and a-actin) from *T. harzianum* produced 1.5, 20.9, 14.8, and 5.8% of informative-char- acters, respectively [Shahid M. *et al*. 2013b, 2014d]. However, taking into account the high taxonomic value of these genes, they are not useful enough for designing strain-specific primers for monitoring *Trichoderma* isolates.

T. harzianum Th azad is commonly used in basic biocontrol research [De la Cruz, J, *et al*. (1999)] and produces a variety of cell-wall-degrading enzymes in natural substrata [Benitez, T. *et al*. (1998)]. Because of its value as a research model, this strain was considered in the present study. In previous work, a strain-specific SCAR marker was developed to distinguish *T. atroviride* 11 from 42 *Trichoderma* spp. strains, including biocontrol agents, belonging to 13 species [Shahid M. *et al*. 2013a, 2014c, Hermosa, MR. *et al*. (2000)]. In the present study, when the SCAR A1 and SCAR A1c primers were tested under annealing conditions at 65°C, A 1.5 kb fragments was amplified with the DNA from *T. harzianum* Th azad. The 1.5-kb band amplified from *T. harzianum* Th azad DNA with SCAR A1 and SCAR A1c primers and sequence analysis provided no evidence of homology with other GenBank sequences.

The specificity of BR1 and BR2 was tested against the DNA isolated from 49 *Trichoderma* species, The primer pair, BR1 and BR2, amplified an 1.5 KB product with *T. harzianum* Th azad DNA. These results suggest that we have identified a suitable molecular marker and that the PCR-specific primers, BR1 and BR2, are useful for detecting *T. harzianum* Th azad. The amount of DNA recovered from a given soil is highly related to factors such as soil type or the method of extraction. In most cases, the recovered DNA from soil is not proportional to the number of inoculated microbial propagules until values are >106 –107 spores per g of soil [Frostegard, A. *et al*. (1999)]. In real-time PCR experiments primers QTh azadf and QTh azad did permit the quantification of a target fragment, contained within the SCAR marker of *T. harzianum Th azad*, from known amounts of spores artificially inoculated in soil samples. The sensitivity of the technique was shown by detecting quantities <0.057 fg of the 1.5kb target fragment of *T. harzianum Th azad*, based on the values of the standard curve. In other real-time experiments using the Taq-Man system, 0.77 fg of *Mycobacterium avium* DNA was detected in fecal samples [Fang *et al*. (2002)]. Our results showed a higher sensitivity (10 times greater) using a Taq-Man probe. In soil sample containing only *T. harzianum Th azad* spores, DNA was quantifiable and the results were reproducible. Fluorescent signals were observed around cycle 31. In the case of soil sample, inoculated with a mixture of 109 spores per 10 g of soil of the 49 strains, including *T. harzianum Th azad*, the results were not consistent or reproducible. This may be explained by the use of a lower annealing temperature, which was determined by the Primer Ex- press program when primers QTh azadf and QTh azadr were designed. In initial experiments, 65°C was the annealing temperature that allowed the specific amplification of the 1.5 kb SCAR marker of *T. harzianum* Th azad, using BR1 and BR2, tested using the DNA from 49 strains. The annealing temperature used in real-time PCR with primers Q Th azadr and QTh azadf primers was 59°C and the total size of the target fragment was

1.5kb. Perhaps under these conditions the primers were nonspecifically binding to sites within the DNA of the various strains included within the mixture and therefore were not able to result in a positive or reproducible fluorescent signal for soil sample, whereas this was not the case for sample containing only *T. harzianum* Th azad. Primers QTh azadf and QTh azadr were also tested using conventional PCR with the DNA from pure cultures of 49 *Trichoderma* spp. Only one amplicon was detected with *T. harzianum Th azad* genomic DNA, from spores, indicating the specificity of Q Th azadf and Q Th azad.

SCAR primers BR1 and BR2 were able to detect *T. harzianum Th azad* using conventional PCR. However, even though QTh azadf and QTh azadr were able to detect *T.harzianum* Th azad in both pure culture and artificially inoculated sterile soil, their specificity was not great enough to detect and quantify *T.harzianum Th azad* among a mixture of strains. These results suggest that the use of real-time PCR to quantify a specific strain within natural soil containing a complex mixture of microbes is not reproducible with these particular primers and conditions. However, this does not mean that this strategy would not be useful to quantify a given species using multicopy genes such as those from the rRNA cluster [Schena, L. *et al*. (2002)].

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