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A multiplex qPCR TaqMan-assay to detect fungal antagonism between *Trichoderma atroviride* (Hypocreaceae) and *Botrytis cinerea* (Sclerotiniaceae) in blackberry fruits using a *de novo* tef1-α- and an IGS-sequence based probes

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ABSTRACT

The aim of this study was to design a *Trichoderma atroviride*-specific qPCR oligo set, evaluate its specificity, and standardize a methodology that quantifies antagonism against *Botrytis cinerea* in blackberry fruits (*Rubus adenotrichos* Schltdl.). Primers and probe were designed based on the nuclear translation elongation factor 1-alpha (tef1-α) of *T. atroviride*. A commercial IGS-based oligo set was used to quantify *B. cinerea.* The specificity of the designed oligo set, along with ITS-based oligo sets, was assessed using other *Trichoderma* species and *B. cinerea*. Multiplex qPCR assays were performed using DNA from *B. cinerea*, *T. atroviride,* and blackberries inoculated with these fungi. Assays with the tef1-α oligo set showed high sensitivity and reproducibility. In inoculated fruits, *T. atroviride* and *B. cinerea* were quantified simultaneously, including in symptomless tissues. This work standardized a qPCR methodology that specifically targets a *T. atroviride* isolate. This newly-designed qPCR oligo set could be useful in future biological control programs.

1. Introduction

Biological control using natural antagonistic microorganisms has been extensively studied with the idea to exploit their potential for biological suppression of plant diseases. *Hypocrea*/*Trichoderma* (Ascomycota, Hypocreales) is a well-studied fungal genus used for biocontrol against numerous plant pathogens [1–9]. Different species of *Trichoderma* have been used for the biological control of *Botrytis cinerea* [10–13], an economically-important pathogenic fungus that affects over 200 plant species without any apparent host specificity [14–17]. In blackberries and raspberries (*Rubus* spp.), *B. cinerea* causes gray mold, one of the most common and serious diseases [17,18] that infects any aerial part of the plant at any stage of development, but particularly infects mature fruit [14,15,19,20].

Many species of the genus *Trichoderma* from around the world have been described through molecular analyses [21]. The diverse survival mechanisms of *Trichoderma*, such as their enzymatic capacity to degrade substrates, versatile metabolism, and resistance to microbial inhibitors, are closely related to their wide distribution and ecological plasticity [5]. The diverse habits of *Trichoderma* spp. include mycoparasitism, antibiosis with secondary metabolites, competition with other fungi for nutrients, saprophytism, endophytism, and induced systemic acquired resistance in host plants [5,21–23]. In Costa Rica, *Trichoderma* isolates from cultivated tropical highland blackberries (*R. adenotrichos* (Schltdl.)) have shown antagonistic activity against *B. cinerea* in laboratory and field evaluations [24–26]. Blackberry growers have shown increasing interest in applying biological control agents like *Trichoderma* in organic production.

Different molecular techniques have been implemented to quantify both phytopathogenic and antagonistic fungi. Real-time polymerase chain reaction (qPCR) is one of these techniques. *B. cinerea* detection and quantification assays using qPCR have been carried out in different plant species [27–35]. For the detection and quantification of *Trichoderma*, primers and probes have been

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designed for different species with the aim to monitor soil-inhabiting strains and biocontrol strains [7,36–39].

Most of the primers and probes designed for the detection and quantification of *Trichoderma* are based on the nuclear ribosomal DNA (rDNA). The rDNA is the most commonly used target region for the identification of many organisms at the species-level because of its highly variable regions, as well as its highly conserved sequences. This region contains the 18S, 5.8S and 28S ribosomal genes separated by the internal transcribed spacers, ITS1 and ITS2, and the intergenic spacer region (IGS). The ITS regions have been extensively sequenced and numerous rDNA reference sequences are currently available in databases, enabling the design of universal primer sets. Numerous sets of oligos have been designed for *Trichoderma* species that amplify the ITS1 and ITS2 regions [7,22,36,39–42], and although the ITS region is considered the barcoding region for fungal identification [22,43–45], differentiation of related species in certain taxonomic groups, such as Hypocreales, is limited due to sequence homology ([23,46–49]; ISTH-International Subcommission on *Trichoderma* and Hypocrea Taxonomy). The translation elongation factor 1-alpha (tef1-α) is a more informative phylogenetic marker, since the gene contains greater sequence variability than the rDNA as well as more informative phylogenetic characters than other regions [3,46,47]. This variability increases the capacity to differentiate between and within closely related groups of *Trichoderma* species [46].

Developing a qPCR probe based on the *Trichoderma* tef1-α gene would be a useful tool to monitor and estimate the efficiency of control of different *Trichoderma* strains against *B. cinerea* on visibly infected or symptomless tissue. The objective of this study was to develop a TaqMan oligo set based on a target *Trichoderma* tef1-α and standardize a multiplex qPCR methodology for the *in planta* fungal quantification of *Trichoderma* and *B. cinerea* on blackberry fruits (*R. adenotrichos*).

2. Materials and methods

2.1. Fungal isolation, purification and inoculation

Blackberry fruits (*R. adenotrichos*, landrace *Vino espina roja*) with visible signs of infection by *B. cinerea* were collected from the district of San Isidro of El Guarco, in the province of Cartago, Costa Rica (N 09°44'39.9" W083°56'15.7"). *B. cinerea* mycelia and conidia from infected fruits were isolated and cultured in Petri dishes on potato-dextrose agar (PDA, Oxoid Ltd., ThermoScientific™) with 25 % lactic acid (PDA + 25LA). Plates were incubated at room temperature (25 °C) in the dark for at least 3 d, purified and recultured in PDA + 25LA. Plates were incubated at 25 °C with an alternating photoperiod of 12 h until *B. cinerea* formed a lawn.

Five fruit-derived *Trichoderma* isolates ([24–26]; Table 1) were reactivated and cultured by following the methodology described above for *B. cinerea*. Pure cultures of *B. cinerea* and *Trichoderma* were obtained by single spore isolation (monosporic cultures) using the methods described by Choi et al. [50]. Plates were left overnight and spore germination was observed within 24 h. Germinating spores were individually selected and transferred onto Petri dishes with PDA + 25LA medium and grown at 25 °C with a photoperiod of 12 h.

Healthy half-ripen (darkish red) blackberries were superficially disinfected in 75 % ethanol for 30 s, 0.5 % NaClO for 1 min and rinsed three times for 1 min in sterile distilled water. Each fruit was placed on a sterile Petri dish with water agar and superficially inoculated with 20 μl of a conidial suspension at a concentration of 1×10^6 conidia/mL. Twenty fruits were inoculated with each fungus (BcLLCR, BV1CR + BcLLCR and BV1CR) for a total of 60 inoculated fruits, and incubated at 25 °C with a photoperiod of 12 h.

2.2. DNA extraction

Total genomic DNA was isolated from monosporic cultures of each fungus (Table 1) following the extraction protocol detailed by Hoyos et al. [51] with some modifications to work with small volumes. Two hundred mg of mycelia and conidia of each fungus were scraped from Petri dishes, transferred to 1.5 mL centrifuge tubes and ground using plastic pestles. Five hundred μl of preheated (60 °C) extraction buffer (50 mM Tris-HCl pH 7,2; 50 mM EDTA; 3 % SDS; 1 M NaCl; 1 % Mercaptoethanol) was added to ground fungal tissue and mixed by inversion. Tubes containing ground fungal tissue were incubated in a 60 °C water bath for 30 min and mixed by inversion every 10 min. One volume (500 μl) of chloroform:isoamyl alcohol (24:1) was added to each tube and shaken to form an emulsion. Tubes were centrifuged at 10,000 xg for 15 min at 4 °C and the supernatant was transferred to a new 1.5 mL sterile tube. This process was repeated. Cold isopropanol (0.54 volumes) was added to each sample and each tube was centrifuged at 10,000 xg for 15 min at 4 °C. The supernatant was discarded and the pellet was washed with 70 % ethanol and dried using a Vacufuge® Plus (Eppendorf). The pellet was resuspended in 200 μl TE buffer (10 mM Tris-HCl, 1 mM EDTA). RNase A was added to a final concentration of 10 μg/mL and the samples were incubated at 37 °C for 1 h. DNA contamination assessment (RNA-free) was verified through electrophoresis in 0.8 % (w/v) TopVision™ (ThermoScientific™) agarose gels stained with 1X GelRed™ (Biotium Inc.) and viewed under UV light (BioDoc-It Imaging™ System).

DNA was extracted from 60 blackberry fruits (1–2 g) inoculated with *Trichoderma* BV1CR and/or *B. cinerea* BcLLCR. Each blackberry fruit was ground using a porcelain mortar and pestle, and DNA extractions were performed following the procedure described by Hoyos et al. [51] without modifications. Additionally, leaf DNA was extracted from *in vitro*-grown blackberry seedlings with the DNeasy Plant Mini kit® (QIAGEN®) following the manufacturer's instructions. RNase treatments were applied as previously described. DNA integrity was verified through electrophoresis on 0.8 % (w/v) TopVision™ (ThermoScientific™) agarose gels stained with 1X GelRed ™ (Biotium Inc.) and viewed under UV light. DNA concentration was determined using standard Lambda DNA (ThermoScientific™) and purity was determined through UV light spectrophotometry with a NanoDrop™2000 (ThermoScientific™) according to the manufacturer's instructions. DNA impurities were removed using DNeasy Mini spin columns from the DNeasy Plant Mini kit®.

Table 1
Trichoderma isolates associated with *Rubus adenotrichos* fruit from different growing regions in Costa Rica used in this study.

Species	Isolate	Location	Georeference	Source
T. asperellum	SM13BCR	San Martín, León Cortés	N 09°43'43.2" W084°00'06.3"	[25]
T. atroviride	BV1CR	Buena Vista, Pérez Zeledón	N 09°30'15.3" W083°39'28.4"	[25]
T. harzianum	BC1CR	Bajo Canet, Tarrazú	N 09°42'01.3" W083°59'49.0"	[26]
T. lentiforme	J2-1CR	Jardín, Pérez Zeledón	N 09°30'08.3" W083°41'29.4"	[26]
T. rifaii	J2-2CR	Jardín, Pérez Zeledón	N 09°30'08.3" W083°41'29.4"	[26]
B. cinerea	BcLLCR	La Luchita, El Guarco	N 09°44'39.9" W083°56'15.7"	Present study

2.3. PCR and DNA sequencing

The ITS region between the internal transcribed spacers, ITS1 and ITS2, of the nuclear ribosomal genes was amplified using the universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3'), ITS2 (5'-GCTGCGTTCTTCTTCATCGATGC-3'), ITS5 (5'-GGAAGTAAAAGTCG-TAACAAGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') [3,40,43,52,53,22,41,42,47]. The third intron and fourth exon of the tef1-α gene was partially amplified with primers EF1-728 M (5'-CATYGAGAAGTTCGAGAAGG-3'), EF-2R (5'-GGARGTACCAGT-SATCATGTT-3'), Ef700f (5'- TCTACCAGTGCGGTGGTA-3') and tef1R (5'-GCCATCCTTCGAACCAGC-3') [3,21,47,54]. These primers were used to amplify DNA from all *Trichoderma* isolates (Table 1), *B. cinerea* and *in vitro*-grown blackberry plants. Twenty five μl PCR reactions were carried out with the following reaction mixture: 1.25 U of DreamTaq™polymerase, 1X DreamTaq™Buffer (ThermoScientific™), 0.2 mM dNTPs, 1 μM of each primer, 50−100 ng of DNA and QIAGEN® nuclease-free water. Reactions were performed in a Veriti™(Applied Biosystems®) thermocycler under the following temperature profile: an initial denaturation step at 95 °C for 3 min, followed by 40 amplification cycles at 95 °C for 1 min, 56 °C for 30 s, and 72 °C for 30 s; and a final extension step at 72 °C for 10 min. The PCR products were analyzed on 1% (w/v) TopVision™ agarose gels stained with 1X GelRed™. Amplicon sizes were determined against a GeneRuler™ (ThermoScientific™) 100 bp DNA ladder. PCR products were sent to Macrogen Inc. (South Korea) for purification and bidirectional Sanger sequencing. Sequences were edited and assembled using BioEdit v.7.2.5 [55]. Basic Local Alignment Search Tool (BLAST®; available at: http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to compare sequences with reference sequences deposited in GenBank® of the National Center for Biotechnology Information (NCBI) for species identification.

2.4. Phylogenetic analysis

The analysis was performed using the tef1-α gene sequences. To calculate genetic relationships and identify species, multiple alignment and cluster analyses of DNA sequences of the *Trichoderma* isolates were carried out. Consensus sequences obtained from the tef1-α were aligned using Clustal W v.2.0 [56], MUSCLE [57] and MAFFT v.7.017 [58], and afterwards were manually refined. The search for the best-fit nucleotide substitution model was performed in jModelTest v.2.1.4 [59] based on Maximum Likelihood (ML; [60]), Bayesian Inference (BI; [61]), Decision Theory calculations (DT; [62]), and the Akaike Information Criterion (AIC; [63]). PhyML v.3.0 [64], RAxML v.7.2.8 ([65]; GTR Gamma model) and Mr. Bayes v.3.2.6 ([66]; "chain length" = 1,100,000, "heat chains" = 4, "burn-in length" = 100,000, "sub-sampling frequency" = 200, "random seed" = 26,500) with 1000 replacements (bootstrap values) were used to generate trees. The K2P substitution model [67] was also used to generate a tree by Neighbor Joining (NJ; 1000 replacements) with Phylogenetic Analysis Using Parsimony (PAUP) v.4.0a (build 165; [68]). In total, 14 GenBank® sequences from *Trichoderma* type material were included in the phylogenetic analysis and compared with the *Trichoderma* isolates in this study (Table 2).

2.5. Primer and probe design

Two primers and a hydrolysis probe (TaqMan®) were designed based on the tef1-α sequence of the BV1CR isolate using BioEdit v7.2.5 [55] and Primer Express® v3.0 from Applied Biosystems®, following the manufacturer's recommendations [72]. The oligo set (primer and probe) encompassed a sequence between the third intron and fourth exon of the tef1-α (92 nucleotides amplicon; Fig. 1) of *T. atroviride* BV1CR (Accession MK644115) (Positions 866–946 of the tef1-α gene of *T. asperellum* NW_020208831.1 genomic DNA used as reference). The probe was fluorescently labeled at the 5'-end with VIC™ and a minor groove binder (MGB) conjugated with a non-fluorescent quencher (NFQ) was attached at the 3'-end. Primers and probe were synthesized and purified by standard desalting and HPLC by Invitrogen™. Both primers and labeled probe had a predicted annealing temperature of 58 °C and 68 °C, respectively.

Primer and probe specificity were evaluated by comparing all the aligned tef1-α sequences from the *Trichoderma* isolates (Table 1), *B. cinerea* and *R. adenotrichos* and by *in silico* PCR tests using an online *in silico* PCR amplification tool (http://insilico.ehu.eus). Publicly available tef1-α sequences of *Trichoderma* accessions from the GenBank were also evaluated for specificity.

2.6. qPCR optimization and data analyses

qPCR assay efficacy was evaluated using the *de novo* designed oligo set and DNA from five *Trichoderma* isolates (Table 1), *B. cinerea* and *R. adenotrichos* leaves. Two oligo sets, based on the ITS regions, designed to detect *T. harzianum* [7], *T. atroviride*, *T. koningii*, and *T. viride* (United States Environmental Protection Agency, n.d.; Table 3) were included in qPCR experiments. A PrimerDesign™ Ltd. Genesig detection and quantification kit was used to quantify *B. cinerea* through qPCR assays. This specific oligo set amplified the IGS region of the nuclear rDNA ([30]; Table 3).

TrichoBV1 F and TrichoBV1 R primer concentrations were evaluated using an optimization matrix with concentrations at 50, 300 and 900 nM. Four reactions were tested for each primer combination with a probe concentration fixed at 250 nM. The concentrations of the Bc3P and TrichoBV1P probes were evaluated in duplicate at 100, 150 and 250 nM, with a primer concentration fixed at 300 nM, following the recommendations of Applied Biosystems® [72]. Standard *B. cinerea* DNA from the Genesig kit and DNA from the BcLLCR and BV1CR isolates (Table 1) were used as positive controls for qPCR. A primer and probe set based on the cytochrome oxidase subunit I (*COX*) [73] was used as a positive internal control to assess DNA quality. *In vitro*-grown, blackberry leaf-derived DNA, and QIAGEN® nuclease-free water were used as negative controls.

A standard curve was constructed based on threshold cycles (Cq) for 10-fold serial dilutions using *T. atroviride* BV1CR and *B. cinerea* BcLLCR DNA, following the recommendations of Applied Biosystems® [74,72] and PrimerDesign™ (n.d.). To do so, triplicate serial DNA dilutions (1:10) from each fungus were prepared. Dilutions ranged from 3×10^5 to 3 copies for the *T. atroviride* haploid genome and from 5×10^5 to 5 copies for the *B. cinerea* haploid genome. Copy number was calculated according to Applied Biosystems® [74] specifications. Using StepOne™ software v2.3 (Applied Biosystems®), a standard curve was generated with the logarithmically-transformed DNA quantities of each dilution series plotted against the Cq values observed, and a linear regression equation was calculated. The efficiency (E) of the PCR was calculated using the equation $E = [10^{(-1/\text{slope})} - 1] \times 100$. Limits of detection (LoD) were calculated using 18 replicates at a 95 % success rate and a 40 cycle cut-off point [75,76].

To evaluate experimental oligo set performance, multiplex manual reactions were prepared and run in duplicate using DNA from fungus-inoculated blackberries at 3 and 10 d after inoculation (DAI) in a StepOne™ real-time PCR thermocycler (Applied Biosystems®), for a total of 60 tested fruits (10 per fungal inoculation per day tested). Each qPCR reaction mixture was 10 μl and included the following: 1X TaqMan® Universal Master Mix II with UNG, (Applied Biosystems®), 300 nM of each primer, 250 nM labeled probes, and 2.5 μl of 1:20, 1:100 or 1:1000 DNA dilutions. Reactions were carried out in MicroAmp™ Fast Optical 48-Well Reaction Plates (0.1 mL) (Applied Biosystems®) covered with

Table 2
GenBank® accession numbers of the isolates used in this study for the phylogenetic analysis and identification.

Accession	Species	Origin	Host/Substrate	Voucher No./Culture	Reference
AF348093.1	*T. afarasin*	Cameroon	Soil	CBS 130755=IMI 393967=G.J.S. 99-227	[47]
FJ463301.1	*T. afroharzianum*	Peru	*Theobroma cacao*	CBS 124620 = G.J.S. 04-186	[47]
GU198294.1	*T. asperelloides*	Vietnam	Soil	G.J.S. 04-111	[69]
AY376058.1	*T. asperellum*	United States	Soil	CBS 433.97	[70]
MK644116	*T. asperellum*	Costa Rica	*Rubus adenotrichos*	SM13BCR	Present study
AF456889.1	*T. atroviride*	Canada	Fungi compost	DAOM 222144	[21]
MK644115	*T. atroviride*	Costa Rica	*Rubus adenotrichos*	BV1CR	Present study
AF348107.1	*T. camerunense*	Cameroon	Soil	CBS 137272=G.J.S. 99-230	[47]
AF348101.1	*T. harzianum*	United Kingdom	Soil	CBS 226.95	[47]
MK644117	*T. harzianum*	Costa Rica	*Rubus adenotrichos*	BC1CR	Present study
AF348099.1	*T. inhamatum*	Colombia	Soil	CBS 273.78 = IMI 287526=G.J.S. 95-39	[47]
FJ463333.1	*T. lentiforme*	Brazil	*Theobroma cacao*	Dis 167e	[47]
MK644114	*T. lentiforme*	Costa Rica	*Rubus adenotrichos*	J2-1CR	Present study
EU338335.1	*T. longibrachiatum*	Canada	Soil	DAOM 166989	[21]
EU248618.1	*T. martiale*	Brazil	*Theobroma cacao*	CBS 123052	[21]
FJ463324.1	*T. rifaii*	Ecuador	*Theobroma gileri*	CBS 130746= Dis 355b	[47]
MK644113	*T. rifaii*	Costa Rica	*Rubus adenotrichos*	J2-2CR	Present study
DQ841726.1	*T. scalesiae*	Ecuador	*Scalesia pedunculata*	CBS 120069 T = G.J.S. 03-74	[71]
AY376053	*T. viride*	Netherlands	Cellulosic tissue	CBS 101526	[21]

CBS = CBS Fungal Biodiversity Centre culture collection, The Netherlands; DAOM = Agriculture and Agri-Food Canada National Mycological Culture Collection; G.J.S. = G.J. Samuels; Dis = H.C. Evans endophyte cultures, IMI = CABI-Bioscience, Egham, U.K.

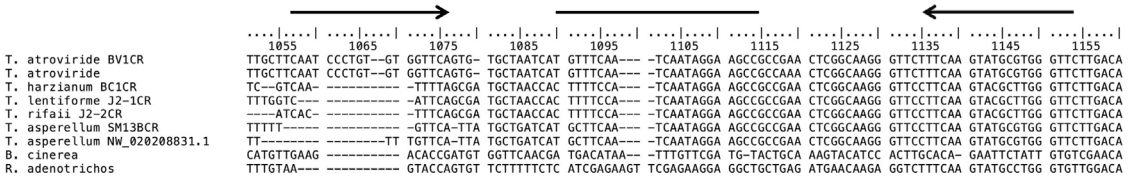

Fig. 1. Multiple alignment of partial sequences of the translation elongation factor 1-alpha (tef1-α), generated by BioEdit, showing the position of the TrichoBV1 F/R primers (arrows) and the TrichoBV1 probe (line) designed to detect *Trichoderma atroviride* isolate BV1CR through qPCR.

Table 3
Primers and probes used in this study for the qPCR assays.

Primers and Probes	Oligo set	Sequence (5'-3')	Target gene	Target species	Source
Bc3 F		GCTGTAATTTCAATGTGCAGAATCC			
Bc3 R	Bc3	GGAGCAACAATTAATCGCATTTC	IGS	*B. cinerea*	[30]
Bc3 Probe[a]		TCACCTTGCAATGAGTGG			
ITS1 S Tharz		TACAACTCCCAAACCCAATGTGA			
ITS1 R Tharz	Tharz	CCGTTGTTGAAAGTTTTGATTCATTT	ITS	*T. harzianum*	[7]
ITS1 TM Tharz Probe[a]		AACTCTTATTGTATACCCCCTCGCGGGT			
TviriF1		CCCAAACCCAATGTGAACCA		*T. atroviride*	
TviriR1	Tviri	TCCGCGAGGGGACTACAG	ITS	*T. koningii*	EPA USA
TviriP1 Probe[a]		CCAAACTGTTGCCTCGGCGGG		*T. viride*	
TrichoBV1 F		TCAATCCCTGTGTGGTTCAG			
TrichoBV1 R	Tatrov	GAACCCACGCATACTTGAAA	tef1-α	*T. atroviride*	Present study
TrichoBV1 Probe[b]		CGGCTTCCTATTGATTGAAACA			

[a] FAM (6-carboxy-fluorescein)-labelled probe at.3'.
[b] VIC (trade name)-labelled probe at.5'.

MicroAmp™ 48-Well Optical Adhesive Films (Applied Biosystems®). The following temperature profile was used: 50 °C for 2 min, 95 °C for 10 min; 50 cycles at 95 °C for 15 s and 55 °C for 1 min with data capture. All Cq values were determined using the StepOne™ software v2.3 software (Applied Biosystems®). Copy number of the target sequences were calculated based on Cq values of the standard curves.

3. Results

3.1. PCR and DNA sequencing

PCR products of approximately 300 bp were generated using the ITS1 / ITS2 primers and of 600–700 bp using primers ITS1 / ITS4, ITS5 / ITS4, EF1–728 M / EF-2R and Ef700f / tef1R for all fungal DNA samples. When BLAST® searches were limited to sequences of type materials (specimens of species registered in taxonomic databases for comparative purposes) deposited in GenBank®, the species identification was different from that obtained in standard searches from non-type material. When compared with GenBank® tef1-α gene sequences of type materials, the BLAST® analysis showed that BC1CR, J2-1CR and J2-2CR were all similar to the species *T. harzianum*, *T. rifaii*, *T. afarasin*, *T. camerunense*, *T. inhamatum*, *T. aggressivum*, and *T. afroharzianum*, while BV1CR was similar to *T. atroviride*, *T. paratroviride*, *T. viride*, *T. scalesiae* and *T. martiale*. The SM13B isolate showed similarity only with *T. asperellum*. BLAST® analysis with ITS sequences showed lower species-level resolution (data not shown) and therefore were not included for phylogenetic analysis.

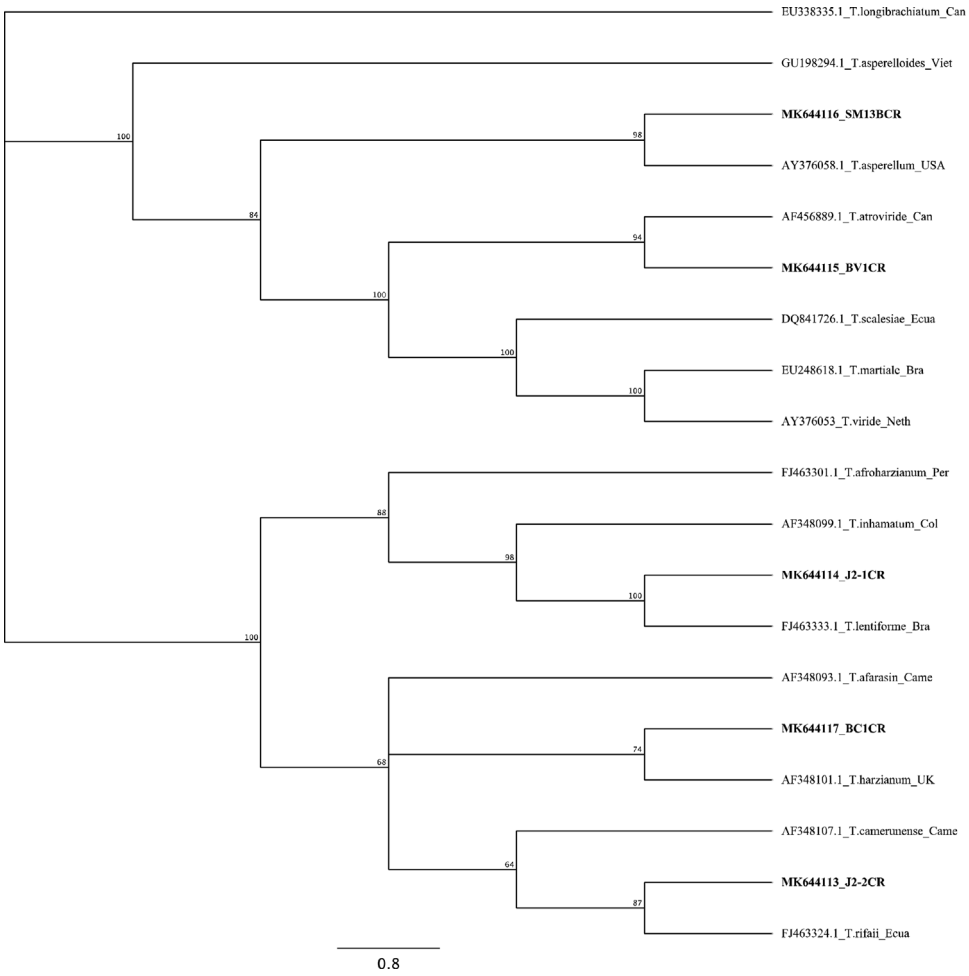

Fig. 2. Phylogenetic tree generated by Mr. Bayes with Bayesian inference based on MAFFT alignment of partial sequences (714 nucleotides) of the tef1-α gene with 1000 replacements, showing the position of different *Trichoderma* species. Sequences from this study and the GenBank® repository are indicated with the corresponding accession number (countries abbreviated as Bra: Brazil, Can: Canada, Came: Cameroon, Col: Colombia, Ecua: Ecuador, Neth: Netherlands, Per: Peru, UK: United Kingdom, USA: United States, Viet: Vietnam; *T. longibrachiatum* used as an external group). J2-1CR, BC1CR, J2-2CR, SM13BCR and BV1CR correspond to the isolates identified in this study (CR: Costa Rica).

3.2. Phylogenetic analysis

The phylogenetic analysis determined species identity and genetic relationships (Table 2 and Fig. 2). The tef1-α sequences from the isolates of the study herein were registered in GenBank® (Table 2). The analysis with jModelTest using the tef1-α gene sequences determined that the best nucleotide substitution model for generating the phylogenetic tree was HKY + I + G with the AIC criterion, HKY + I with the BI, DT criteria, and JC with the ML criterion (Supplementary Table 1). Therefore, the model HKY + I (-lnL = 3564.3774) was selected for inferring dendrograms with all programs. The tree generated by Mr. Bayes with MAFFT showed the highest bootstrap values (Fig. 2). The BV1CR (MK644115) isolate grouped with *T. atroviride* (AF456889), SM13BCR (MK644116) with *T. asperellum* (AY376058), J2-1CR (MK644114) with *T. lentiforme* (FJ463333), J2-2CR (MK644113) with *T. rifaii* (FJ463324) and BC1CR (MK644117) clustered with *T. harzianum* (AF348101) (Fig. 2). The tree generated by NJ with PAUP had a similar topology to that generated by Mr. Bayes (data not shown).

3.3. Primer and probe design, qPCR optimization and data analyses

The designed oligo set in the present study (TrichoBV1 F/TrichoBV1 R/TrichoBV1 P) detected and quantified the BV1CR isolate using total genomic DNA from monosporic cultures and in composite plant tissue samples infected with *B. cinerea.* The DNA was quantifiable and the results were reproducible after all standardizations. Positive detection curves were obtained at all primer and probe concentrations and yielded similar Cq values (Figs. 3 and 4). The 300 nM concentration was chosen for both primers (*forward* and *reverse*) and 250 nM for each probe, which produced intermediate Cq and standard deviation values (Figs. 3 and 4).

The tef1-α-based oligo set showed no detection curves with DNA from *R. adenotrichos* leaves, BcLLCR (*B. cinerea*), BC1CR (*T. harzianum*) or SM13BCR (*T. asperellum*). Although there was no product amplification in *in silico* PCR tests, isolates J2-1CR (*T. lentiforme*; Cq = 40.14) and J2-2CR (*T. rifaii*; Cq = 44.70) showed late detection curves (>40 cycles; Table 4). The primer and probe set designed by Suárez et al. [30] specifically detected genomic DNA from the BcLLCR isolate without compromising results in the presence of the TrichoBV1 probe in multiplex reactions. The primer and probe set designed by López-Mondéjar et al. [7]; (ITS1 S Tharz/ITS1 R Tharz/ITS1 TM TharzP), based on a *T. harzianum* ITS region, generated early detection curves when DNA from BC1CR (*T. harzianum*; Cq = 25.76), J2-1CR (*T. lentiforme*; Cq = 27.08), and J2-2CR (*T. rifaii*; Cq = 25.57) were used, and late detection curves when BcLLCR DNA (*B. cinerea*; Cq = 36.82) was used (Table 4). The ITS-based Tviri oligo set showed late detection curves (Cq > 38.40) with DNA from BV1CR (*T. atroviride*) and *B. cinerea* (Table 4).

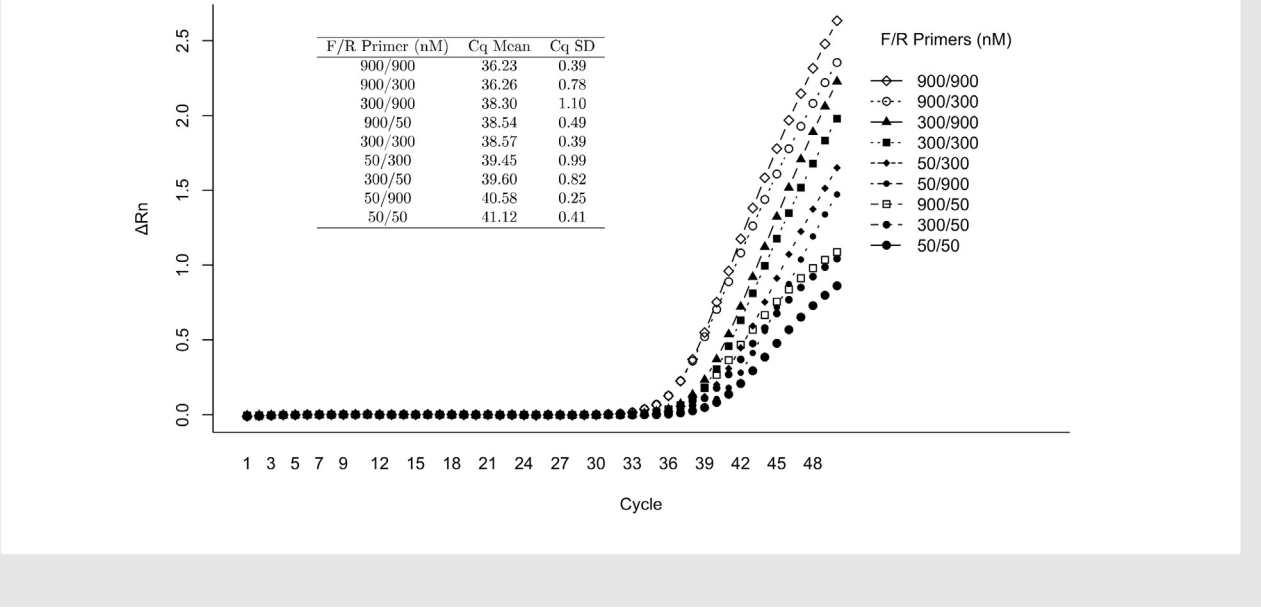

Fig. 3. Evaluation of nine primer concentration combinations of the primers TrichoBV1 F and TrichoBV1 R with 250 nM of TrichoBV1 probe, showing the average Cq of four reactions of each primer combination (50/50, 50/300, 50/900, 300/50, 300/300, 300/900, 900/50, 900/300, 900/900 nM) and its standard deviation (SD). F: *forward*, R: *reverse*.

Multiple ITS sequence alignment showed that both ITS-based primer-probe sets had identical complementary sequences with those species (data not shown). Tatrov primer sequences showed high specificity with tef1-α gene sequences from other *T. atroviride* accessions from GenBank® (Supplementary data).

There was partial or total inhibition in the BV1CR standard curve (Fig. 5), where the highest concentration tested (3×10^5 copies of the DNA of interest) showed the highest Cq values instead of showing the lowest values (Supplementary Table 2). This type of inhibition was also observed when DNA from inoculated fruit samples with visible presence (total fruit colonization) of *Trichoderma* BV1CR was tested. When 1:20 and 1:100 DNA dilutions were used in the qPCR reaction mixture, no curves were detected (no quantification data could be obtained from BV1CR DNA) or Cq values were reported after 40 cycles of qPCR (Supplementary Table 3). In the case of the Bc3 probe for *B. cinerea*, there was no inhibition and Cq values from all dilutions of the standard curve were accepted (Fig. 6). The LoD (95 % success) for *T. atroviride* BV1CR was 300 copies and for *B. cinerea* was 50 copies of the target sequence. Both standard curves showed a high correlation between the Cq values and the absolute DNA quantities of each fungus (R^2 = 0.91, *T. atroviride* BV1CR, R^2 = 1, *B. cinerea*), and high reaction efficiencies; 98.80 % for *T. atroviride* BV1CR and 94.71 % for *B. cinerea* (Figs. 5 and 6). The coefficient of variation (CV) was 4 % and 5 % for the Tatrov and the Bc3 oligo set, respectively.

Multiplex qPCR assays reliably quantified both *B. cinerea* BcLLCR and *T. atroviride* BV1CR in composite samples of both dual-inoculated and single-inoculated blackberry fruits at 3 and 10 DAI (Table 5). Multiplex qPCR assays quantified *Trichoderma* and *Botrytis* DNA in the presence or absence of any visible signs of fungal growth on fruit surfaces (hyphae or spores). *B. cinerea* was detected in fruits even when fruits were not artificially inoculated, which implies that *B. cinerea* was present within blackberry drupelets or other internal structures. The Tatrov probe specifically detected *T. atroviride*; *B. cinerea* was not detected with the Tatrov oligo set. The Bc3 oligo set detected *B. cinerea* at higher quantities at 10 DAI, except in dual inoculations. When *B. cinerea* and *T. atroviride* were co-inoculated, there was a reduction of *B. cinerea* at 3 DAI. Also, *T. atroviride* was detected in greater quantity at 10 DAI while *B. cinerea* was detected in smaller quantities. High SD about the mean Cq values were observed in fruit samples (up to 7.94) (Table 5), more so than in DNA samples extracted from monosporic fungal lawns.

4. Discussion

4.1. Species identification and sequence analysis

According to Chaverri et al. [3], the DNA sequence analysis of ITS region and three genes (α-actin, calmodulin, and tef1-α) of *T. harzianum* demonstrated that each gene had a different percentage of informative characters (1.5, 5.8, 14.8 and 20.9 %, respectively), where the ITS was the least informative. Nevertheless, sequencing the ITS region is still routinely used to provide preliminary identification of *Trichoderma* at the genus level [23,47,48]. Therefore, this region was also sequenced in the present study as a pre-screen (data not shown). The tef1-α was a much more informative genetic marker in the identification and phylogenetic analysis of *Trichoderma* isolates.

In this study, the objective was to design a specific probe that would detect a *T. atroviride* isolate. Thus, using the tef1-α as a target sequence was a more appropriate choice for designing a qPCR primer and probe set.

4.2. Assay optimization

We tested different primer and probe concentrations in multiplex reactions for the simultaneous quantification of *B.*

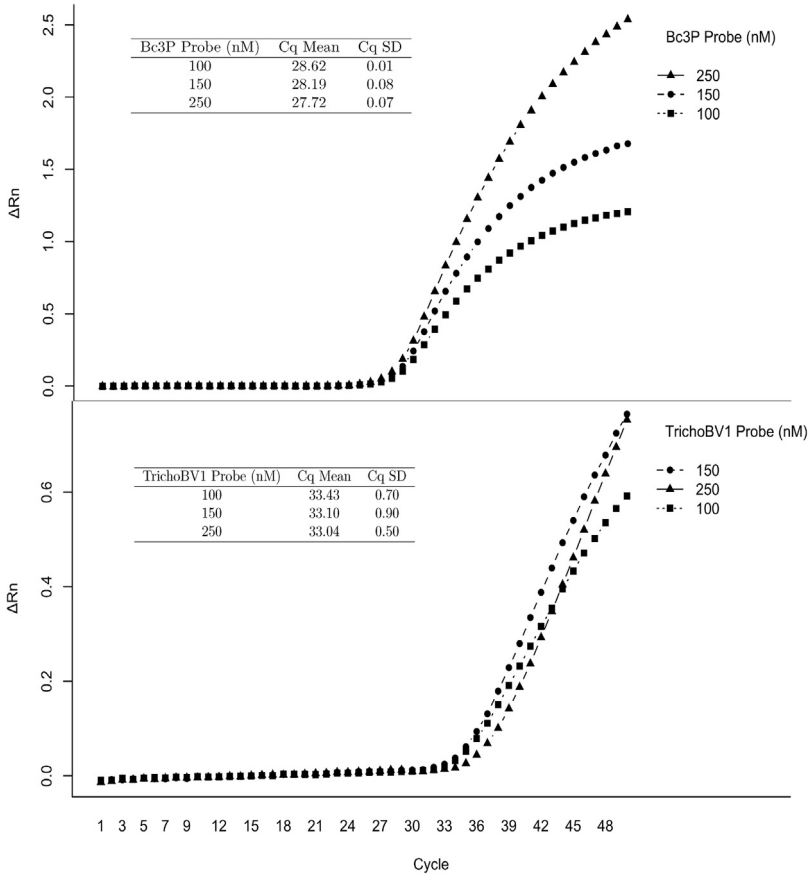

Fig. 4. Evaluation of three concentrations of Bc3 and TrichoBV1 probes with 300 nM of its corresponding primers, showing the average Cq of each probe concentration and its standard deviation (SD). Bc3P probe for *B. cinerea* and Tricho BV1 probe designed for *Trichoderma* BV1CR isolate.

Table 4
Isolates and primer-probe sets evaluated by qPCR in the present study.

		Mean Cq			
Accession	Species	Bc3	Tharz	Tviri	Tatrov
SM13BCR	*T. asperellum*	–	–	–	–
BV1CR	*T. atroviride*	–	–	38.40	29.70
BC1CR	*T. harzianum*	–	25.76	–	–
J2-1CR	*T. lentiforme*	–	27.08	–	40.14
J2-2CR	*T. rifaii*	–	25.57	–	44.70
BcLLCR	*B. cinerea*	24.30	36.82	43.87	–

cinerea BcLLCR and *T. atroviride* BV1CR. Detection of target sequences can be inhibited when one of the target genes is present in a greater quantity than the other [72]. In our case, the *Botrytis* IGS region is present in greater abundance than the tef1-α gene, however, the primer and probe concentrations had little effect on Cq detection values (Figs. 3 and 4). We decided to work with the highest probe concentration tested (250 nM) and intermediate primer concentration 300 nM for both target fungi, for single and multiplex reactions.

High concentrations of *Trichoderma* BV1CR DNA inhibited the Tricho BV1P probe's performance. We were able to discard the possibility of competition between the *B. cinerea* probe and the *T. atroviride* probe because the effect was observed both in multiplex and in single reactions with the TrichoBV1P probe. According to Keer [77], in some experiments, the fluorescent signal may decrease after the exponential phase. This is known as the "hook effect" because of the shape of the curve generated. This is caused by the competitive hybridization that occurs between the single

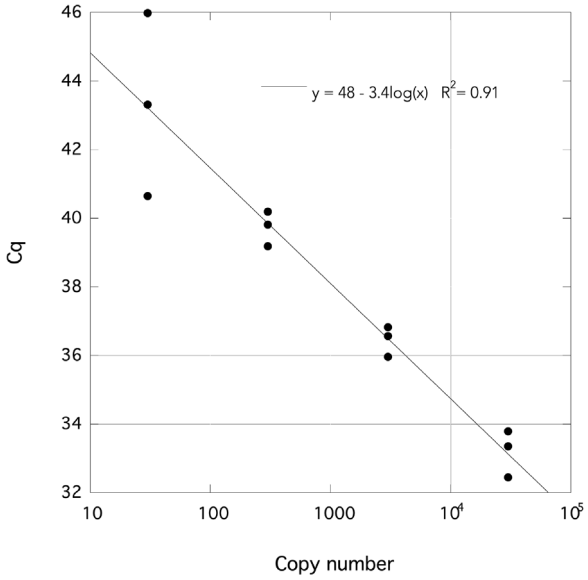

Fig. 5. Standard curve for absolute quantification of *T. atroviride* BV1CR isolate based on the amplification of DNA serial dilutions (1:10), with the Tatrov oligo set (TrichoBV1 F/TrichoBV1 P/ TrichoBV1 R). The efficiency of the reaction was 98.80 % (Y-intercept = 48.141; Slope= -3.351). The limit of detection (LoD-95 %) was 300 copies of BV1CR DNA.

strands of the PCR product and the probe, and we attribute the inhibition of Tricho BV1P probe to this phenomenon. At a low product concentration, the probe competes effectively and therefore fluoresces. However, when the amount of PCR product

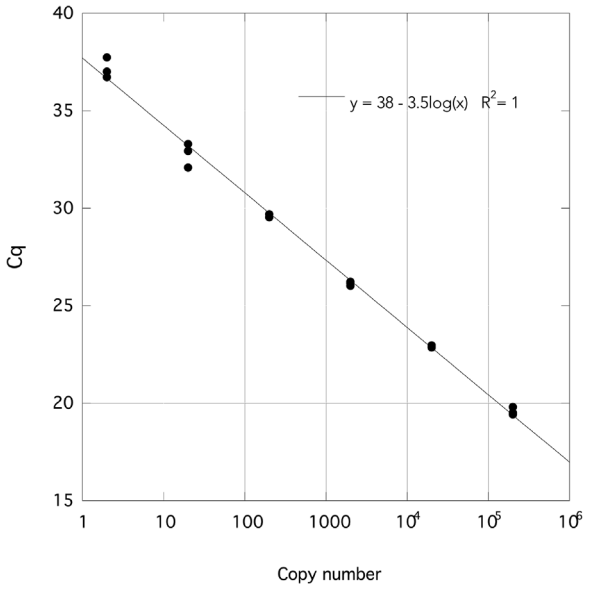

Fig. 6. Standard curve for absolute quantification of *B. cinerea* BcLLCR isolate based on the amplification of DNA serial dilutions (1:10), with the Bc3 oligo set (Bc3 F/Bc3 P/ Bc3 R). The efficiency of the reaction was 94.71 % (Y-intercept = 37.709; Slope= -3.456). The limit of detection (LoD-95 %) was 50 copies of BcLLCR DNA.

Table 5
Mean DNA quantifications using the Bc3 (Bc3F/P/R) and the Tatrov (TrichoBV1F/P/R) oligo sets based on 1:1000 DNA dilutions from 60 blackberries single-inoculated or dual-inoculated with *B. cinerea* BcLLCR, and/or *T. atroviride* BV1CR, in duplicate qPCR multiplex reactions. DAI: days after inoculation; SD: standard deviation.

Oligo set	Inoculation treatment	DAI	Mean Cq	SD	Target copy No.
Bc3	*T. atroviride*	3	35.31	5.21	6
Bc3	*T. atroviride*	10	32.27	2.28	43
Bc3	*B. cinerea*	3	35.57	4.12	5
Bc3	*B. cinerea*	10	33.25	7.94	23
Bc3	*T. atroviride* + *B. cinerea*	3	32.75	2.12	32
Bc3	*T. atroviride* + *B. cinerea*	10	35.90	2.50	4
Tatrov	*T. atroviride*	3	46.20	6.10	3
Tatrov	*T. atroviride*	10	31.13	2.48	91,572
Tatrov	*B. cinerea*	3	46.49	7.86	3
Tatrov	*B. cinerea*	10	50	0	0
Tatrov	*T. atroviride* + *B. cinerea*	3	38.44	7.11	648
Tatrov	*T. atroviride* + *B. cinerea*	10	35.60	7.61	4437

is high, the two single strands of the PCR product bind to each other more quickly than the probe to its target sequence or binding site, so that the amount of fluorescence emitted decreases. According to Keer [77], this decrease in fluorescence does not affect the efficiency or specificity of the amplification or detection of the target DNA and this effect can be avoided by optimizing the DNA concentration in the reaction or by reducing the number of amplification cycles. In the case of *Trichoderma* BV1CR, the detection of the target DNA was affected and a late detection of the fungus was observed (high Cqs) or there were no amplification curves (in fruits completely covered with the fungus) when qPCR reactions were run using highly concentrated DNA. Adjusting the sample dilutions to 1:1000 allowed the expected detection and quantification of *Trichoderma* BV1CR present in the samples.

4.3. Primer and probe set performance

In *Botrytis*, the ITS regions also had limited species resolution, and therefore other regions of the genome have been studied for species identification. The IGS region between the ribosomal genes has evolved faster than the ITS regions and has more sequence variation, thus the IGS region is a much more useful tool to differentiate between closely related species [30,78,79]. The capacity of the IGS region to discriminate among species explains the high fidelity and specificity of the qPCR probe designed by Suárez et al. [30] to quantify *B. cinerea*. That probe successfully quantified *B. cinerea* in multiplex and single probe assays using total DNA extracted from the BcLLCR isolate and from composite biological matrices in the present investigation, and has been used by other researchers to study this pathogen applying qPCR technology [33,34,79].

In the case of *Trichoderma*, generic probes, as well as taxon-specific probes, have been developed to monitor and quantify natural populations or strains inoculated in soils or biological matrices [7,36,37,39]. All of these studies demonstrated the effectiveness and efficiency of the qPCR technique, as well as its applicability in fungal detection and monitoring programs. However, those probes were designed based on regions with little interspecific differentiation, such as the ITS, thus high specificity was not possible. In the present study, the ITS-based probe reported by López-Mondéjar et al. [7] (Tharz; Table 3) amplified and yielded low Cq values when DNA from three different *Trichoderma* species were used and mid Cq values when DNA from *B. cinerea* was used (Table 4); *T. harzianum* (BC1CR), *T. lentiforme* (J2-1CR) and *T. rifaii* (J2-2CR). Likewise, the EPA oligo set (Table 3) had low affinity for one of the target species (*T. atroviride*) and low affinity for a non target species (*B. cinerea*) (Table 4). The low specificity of the aforementioned probes is explained by the similarity of the ITS region among all these species. Those oligo sets should not be used to monitor and quantify *B. cinerea* and *T. atroviride* inoculated on blackberry fruits because Cq values would be confounded by both fungal DNAs.

The Tatrov oligo set demonstrated high specificity and affinity for *T. atroviride* BV1CR DNA in qPCR assays and was the only set of oligos that did not generate detection curves when *B. cinerea* DNA was added (Table 4). The Tatrov oligo set yielded detection curves with J2-1CR and J2-2CR DNA with Cq values above 40 cycles in qPCR, although no amplification occurred in *in silico* PCR tests. Nonetheless, Cq values for the target species, *T. atroviride*, were much lower (Table 4). This means that the probe quantified DNA from all three species, but with higher affinity when using *T. atroviride* DNA and to a much lesser extent when *T. lentiforme* or *T. rifaii* DNA was present in qPCR assays. Multiplex and single reactions should have a cut off at 40 reaction cycles when using the Tatrov oligo set for reliable quantification.

In multiplex reaction experiments, the Tatrov probe's performance and the data collection were not affected by the presence of other *Trichoderma* fungi because blackberry fruits were inoculated with the BV1CR (*T. atroviride*) isolate and *B. cinerea*. Multiplex reactions using both sets of oligos, Bc3 and Tatrov, can reliably quantify each organism separately in composite samples (Table 5). Fruit assays demonstrated that *B. cinerea* is present within blackberry fruits and cannot be removed by surface sterilization because *Botrytis* remains latent during early floral and fruit development [18,80–82]. Interpreting biological interactions between *B. cinerea* and *T. atroviride* on or within blackberry fruits requires a greater number of samples. However, the results demonstrate the probes' capability to detect the target fungus within a complex matrix.

The characterization and identification of isolates from fungi and other organisms at the species level is essential for their study and use as biological control agents. This requires methods that monitor their presence or absence in the environment in which they are used. Traditionally, culture techniques have been used in the laboratory through selective media and morphological and morphometric identification. However, this method takes time, does not always distinguish between or among species with similar morphologies and does not allow quantitative or direct

tissue analysis. Real-time PCR with hydrolysis probe technology (TaqMan®) is an effective method to monitor and quantify organisms of interest present in simple or complex matrices, and is a useful technique when working with biological control agents. The qPCR assays performed in this investigation were sensitive and showed fast, selective and consistent results with composite DNA samples. Our findings outline and demonstrate the development of a useful tool for studying fungi that grow on or inside plant tissues and in symptomless hosts.

The results of this study have established the guidelines of a standardized, sensitive, multiplex methodology for evaluating the action of *T. atroviride* BV1CR as an antagonist of *B. cinerea* on blackberry fruits. We have developed a specific probe that selectively detects *T. atroviride* and does not detect *B. cinerea.* To our knowledge, this is the first report of a qPCR probe based on a powerful barcode marker, the tef1-α DNA sequence, for the detection of *Trichoderma* [83]. We propose the use of the Tatrov oligo set for mycological studies, competition assays, biological control programs and field evaluations with *T. atroviride*. The oligo set can also be used to evaluate active formulations of biopesticides.

CRediT authorship contribution statement

Irena Hilje-Rodríguez: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing, Visualization. **Federico J. Albertazzi:** Conceptualization, Formal analysis, Data curation, Writing - original draft, Writing - review & editing, Visualization. **German Rivera-Coto:** Conceptualization, Funding acquisition. **Ramón Molina-Bravo:** Conceptualization, Formal analysis, Writing - original draft, Writing - review & editing, Visualization, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.btre.2020.e00447.

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