DEVELOPMENT OF CONVENTIONAL AND REAL TIME PCR ASSAY FOR THE RAPID DETECTION AND QUANTIFICATION OF A BIOCONTROL AGENT, *CHAETOMIUM GLOBOSUM*

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SUMMARY

Chaetomium globosum is a potential biocontrol agent against various seed and soil-borne pathogens. To ensure proper use of *C. globosum* in agriculture, accurate data is essential for population monitoring. A PCR-based marker has been developed for detection of this biocontrol agent, which will help to detect the fungus at the place of its application. Out of twelve URP primers tested against 15 isolates of *C. globosum* and other *Chaetomium* species, URP 2R amplified a monomorphic band of 1,900 bp only in *C. globosum* isolates. This amplicon was cloned and sequenced, and based on the sequence obtained, four primer sets were designed, one of which in PCR assays amplified a region (SCAR; $SCCgRA_{1900}$) of the expected size (1.9 kb) in *C. globosum* isolates. The specific marker also detected the presence of *C. globosum* in soil, roots and leaves. The detection limit of marker in conventional PCR assay was 75 pg. The sensitivity and usefulness of SCAR marker was further enhanced by developing qPCR using the primer set SCCgQF/SCCgQR designed from $SCCgRA_{1900}$, which detected as much as 1 pg of DNA (4.83×10^5 copy number of target DNA). The initial population of *C. globosum* in terms of target DNA in *C. globosum*-amended soil was equivalent to 2.5×10^8 copy number/g soil (0.51 ng target DNA/g soil) which increased approximately 10 times after 15 days of application i.e., 2×10^9copy number/g soil (3.1 ng/g soil). However, with, *Bipolaris sorokiniana* the quantity of *C. globosum* target DNA increased slowly reaching 4.32×10^8 copy number/g soil after 15 days. Conventional PCR-based detection using SCAR marker and subsequent qPCR provided a rapid and reliable tool for efficient detection and monitoring of *C. globosum* at the site of its application.

Key words: biocontrol agent, *Chaetomium globosum*, SCAR marker, qPCR.

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INTRODUCTION

The application of fungal biocontrol agents (BCAs) is becoming an increasingly important alternative to chemicals in protecting crops against weeds, insects and fungal pathogens in both agriculture and forestry (Tyler *et al*., 2001). The population dyanamics of biocontrol agents in the field need to be well understood for their successful exploitation (Lo *et al*., 1996). *Chaetomium* is a genus of the class Pyrenomycetes (Ascomycotina), order Sordariales and family Chaetomiaceae. It is a common colonizer of soil and cellulose-containing substrates and has been reported to be a potential biocontrol agent. *C. globosum* is effective against seed rot and damping off caused by several seed- and soil-borne plant pathogens like *Pythium ultimum*, *Alternaria raphani*, *A. brassicicola* and *Fusarium* spp. (Harman *et al*., 1978; Vannacci and Harman, 1987). *C. globosum* is also antagonistic to the rice blast pathogen (*Pyricularia oryzae*) (Tyler *et al*., 2001). Our earlier studies have shown its effectiveness in controlling spot blotch of wheat caused by *Cochliobolus sativus* (Aggarwal *et al*., 2004) and Ascochyta blight of chick pea (Rajkumar *et al*., 2005). Biochemical characterization of the fungus has shown the production of xylanase and β 1,3-glucanase (Ahammed *et al*., 2008, 2012). Besides hydrolytic enzymes, *C. globosum* is also known to produce various metabolites such as chaetomin, chaetoglobosin and BHT (Biswas *et al*., 2012; Di Pietro *et al*., 1992) having antifungal properties against phytopathogenic fungi. The colonization and distribution of agents in soils are important features for evaluating their exploitability. The fate of commercial biocontrol agents needs to be monitored by developing strain- and species-specific markers which can also track their population over time (Savazzini *et al*., 2008).

Several methods have been established to quantify fungi in soils, including counting the number of fungal spores physically or by plating on selective medium. These techniques have limited success, since they neither ensure adequate sensitivity and accuracy, nor differentiate the desired species isolate from the native organisms. Compared to the above methods, the use of molecular markers provides much promise for the rapid identification and quantification of specific biocontrol agents in soil and plant. The genome of a microorganism offers several

possibilities for monitoring. Fungal isolates which carry a functional gene can be targeted through amplification of the sequence (Mavrodi *et al*., 2007; Xiang *et al*., 2010). Other sequences, such as fungal internal transcribed spacer (ITS) and intergenic spacer (IGS) regions can be targets for detection of specific microorganisms. Sequences with unknown function can also be utilized for detection purposes. There are many approaches to identify these intrinsic markers, which may distinguish between species or even strains within a species. Universal rice primer (URP)/ random amplified polymorphic deoxyribonucleiotide primers (RAPD) are useful for the characterization and grouping of isolates in order to study their genetic relatedness (Aggarwal *et al*., 2008, 2010; Bulat *et al*., 1998; Lubeck *et al*., 1999). URP/RAPD primers primarily target intergenic, more variable areas of the genome and therefore are more suitable for detection of interspecific variations. These primers amplify variable genome regions resulting in a DNA fingerprinting. By comparing fingerprints of related species, PCR products singling out one species, can be identified and sequenced for development of species-specific SCAR (sequence-characterized amplified region) markers (Aggarwal *et al*., 2012; Becker *et al*., 1999; Suarez *et al*., 2005).

SCAR markers are useful for the detection of specific microorganisms in microbial systems, and their use is further supported by real-time PCR (Raeymaekers, 1998). Compared to conventional PCR, real-time PCR has significant advantages. This technique does not require post-amplification processing steps and hence reduces the time and labour and is greatly suitable for large-scale analyses (Schena *et al*., 2002, 2013). This method is highly sensitive, with reliable detection of even 1 pg or less fungal DNA in treated soil or plant tissues.

The aim of the present study was to develop and validate endogenous SCAR marker that allow the detection, diagnosis and monitoring of *C. globosum* in soil and in *planta*. In addition, to develop a qPCR based analysis to facilitate accurate, rapid, reliable and very sensitive quantification system for this biocontrol agent at the site of its application and to study its population dynamics during interaction with pathogens.

MATERIALS AND METHODS

Fungal isolates. Fifteen isolates of *C. globosum*, and one isolate of each *C. reflexum*, *C. cochlioides*, *C. perlucidum* and *C. cupreum*; other pathogens such as *Aspergillus flavus*, *Bipolaris sorokiniana*, *Fusarium moniliforme*, *Alternaria triticina*, *Tilletia indica*, *Epiccocum purpurecens*, *Rhizoctonia solani*, the biocontrol agent *Trichoderma virens* and wheat yellow rust (*Puccinia striiformis*) were taken from culture collection of our laboratory at the Indian Agricultural Research Institute, New Delhi (Table 1). All culturable fungi were maintained on PDA (potato dextrose agar) slants and uredospores of *P. striiformis* (yellow rust race 78S84) were propagated on wheat seedlings of cv. Agralocal. The fungal isolates were grown in 100 ml potato dextrose broth [20% potato, 2% dextrose (w/v)] placed in a 250 ml flask for 5 days at 24 ± 2°C with 12 h photoperiod and 100 rpm shaking in shaker incubator (Kuhner, Biogenetek, India). The mycelia of all fungal species were harvested and separated by filtration through Whatman no.1 paper, washed thrice with sterile water, air-dried and stored at −40°C until extraction of DNA. Pure samples of uredospores of yellow rust race were collected and stored at −40°C until DNA extraction.

DNA extraction from the biocontrol agent, other fungi, wheat leaves and soil samples. DNA was extracted from all fungal isolates using the cetyltrimethyl ammonium bromide method (Murray and Thompson, 1980) with slight modifications. The mycelium (5 g) was finely ground in liquid nitrogen and 20 ml of DNA extraction buffer (0.1 M Tris, 1.5 M NaCl, 0.01 M EDTA, 2% CTAB and 0.2% β-mercaptoehanol) was added to the powder. The suspension was incubated at 65°C for 1 h with occasional stirring. An equal volume of chloroform:isoamyl alcohol (24:1) was added to each tube followed by centrifugation. The upper aqueous phase so obtained was precipitated with 0.1 vol. of 3 M sodium acetate and 0.6 vol. of ice-cold isopropanol. After precipitation, tubes were centrifuged at 11,000 rpm for 10 min at 4°C. The pellet was washed with 70% ethanol and dried at room temperature. Finally, the nucleic acid was dissolved in TE buffer (10 mM Tris HCl, 1 mM EDTA; pH 8.0).

DNA was also extracted from inoculated and uninoculated wheat leaves, soil samples as well as from uredospores of yellow rust. ZR Soil Microbe DNA Kit™ (Cat. no. D6001; Zymo Research Corp., USA) was used for quick DNA extraction from these samples. DNA from different samples was quantified using nanodrop and stored at –20°C.

PCR amplification of *Chaetomium* spp. using URP primers. All *Chaetomium* spp. isolates were used for PCR amplification using 12 URP markers. The URP-PCR reactions were performed in 25 µl vol. containing 75 ng of genomic DNA of each isolate, 200 µM each dNTP (dATP, dGTP, dCTP and dTTP), 0.2 µM primer, 1.5 mM $MgCl_2$, 2.5 U Taq DNA polymerase and 1× Taq buffer. The thermal cycler was programmed for one cycle of denaturation at 94°C for 4 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min. A final extension step at 72°C for 7 min was also done. Sterilized distilled water was used as negative control in every experiment to test the presence of contamination in PCR reagents. URP markers which gave monomorphic bands with the 15 isolates of *C. globosum* were further used for PCR amplification along with one isolate each of *C. reflexum* and *C. perlucidum*.

Table 1. Details of isolates of different fungi used to screen primer specificity

S. No.	Designated name of isolates as per ITCC*	Source	Location
1	*Chaetomium globosum* 1627 (Cg1)	Coprophilous	IARI farm, New Delhi, India
2	*C. globosum* 6210 (Cg2)	Wheat leaves	IARI farm, New Delhi, India
3	*C. globosum* 2401 (Cg3)	Dolichos seed	Nainital, Uttaranchal, India
4	*C. globosum* 2034 (Cg4)	Wheat grains	IARI, New Delhi, India
5	*C. globosum* 6215 (Cg5)	Wheat leaves	IARI farm, New Delhi, India
6	*C. globosum* 6211 (Cg6)	Wheat leaves	IARI farm, New Delhi, India
7	*C. globosum* 6214 (Cg7)	Wheat leaves	Dhaulakuan, HP, India
8	*C. globosum* 6218 (Cg8)	Wheat leaves	Samastipur, Bihar, India
9	*C. globosum* 6212 (Cg9)	Wheat leaves	Jammu, J&K, India
10	*C. globosum* 6220 (Cg10)	Wheat leaves	Dhaulakuan, India
11	*C. globosum* 6216 (Cg11)	Wheat leaves	Pantnagar, India
12	*C. globosum* 6221 (Cg12)	Wheat leaves	Jaipur, India
13	*C. globosum* 6219 (Cg13)	Wheat leaves	IARI, India
14	*C. globosum* 6213 (Cg14)	Wheat leaves	Pune, India
15	*C. globosum* 6217 (Cg15)	Wheat leaves	Jammu, India
16	*C. reflexum* 5002	Insect	CPCRI, Kasargod, India
17	*C. perlucidum* 6009	Bottle palm	Dept. of Pl. Path., Junagadh, Gujrat, India
18	*C. cochlioides* 3326	Wheat leaves	Delhi University, New Delhi, India
19	*C. cupreum* 4600		Godhra, India
20	*Aspergillus flavus* 5076	Soil	Hyderabad, India
21	*Bipolaris sorokiniana* 5439	Wheat	Karnal, India
22	*Fusarium moniliforme* 4412	Sugarcane	Navsari, India
23	*Alternaria triticina* 5125	Wheat	Delhi, India
24	*Tilletia indica*	Wheat	Delhi, India
25	*Epiccocum purpurecens* 3673	Maize leaf	Delhi, India
26	*Puccinia striiformis* (STK)	Wheat	Shimla, India
27	*Trichoderma virens* 4911	Soil	Barrcakpore, India
28	*Rhizoctonia solani* 5308	Maize	Delhi, India

* ITCC-Indian Type Culture Collection

PCR products were electrophoresed in 1.2 % (w/v) agarose gel containing ethidium bromide (0.5 µg/ml) in 1× Tris-acetate EDTA buffer.

Cloning and sequencing of unique amplicon. Amplified DNA fragments were separated on 1.2% agarose gel. The species-specific band was purified from the gel using agarose gel extraction kit (Thermo Scientific GeneJET Gel Extraction Kit) and cloned into pGEM-T easy vector (Promega, USA). Ligations, transformations of *Escherchia coli* strain XLBlue and plasmid amplifications were performed following standard procedures (Sambrook and Russell, 2001). After cloning, 20 positive colonies were selected and each of them was cultured overnight in Luria-Bertani (LB) liquid medium containing ampicillin (100 mg/l). The size of the cloned fragment was verified by using the corresponding URP primer and digested by restriction enzymes in the multiple clone site of vector. The recombinant plasmids were extracted using a commercial kit (Nucleopore, Genetix Bio, India) and fragments were sequenced with an automated DNA sequencer (Model 3100 version 3.0ABI Prison) at the South Campus of the University of Delhi, India. The sequences were assembled using Bioedit V7.05 version.

Design of SCAR primers and optimization of PCR conditions. Primers for candidate SCAR markers were designed using Primer-3 software (website: http://www.genome.wi.mit.edu/genome_software/other/primer3.html). The primers were compared with the sequences in the database through BLAST and FASTA searches to confirm their specificity and their design was optimized using the Net Primer software (Biosoft International, www. Primer biosoft.com/ net primer.html). Different sets of primers were synthesized by Xceleris (New Delhi, India). PCR amplification was carried with designed primer pairs and PCR reaction mixes were same as given earlier. However, PCR conditions were standardized using gradient PCR with denaturation at 94°C for 30, 40, 50 sec, annealing at 55, 62, 68°C for 30, 40, 50 sec and extension at 72°C for 40 and 90 sec for obtaining optimal amplification. A final extension step was at 72°C for 7 min. The primer pair and PCR conditions that yielded the amplification of a distinct band only with *C. globosum* was selected for further studies. All PCR reactions with designed primers were repeated at least three times. The PCR products were checked on agarose gel as described above.

Specificity and sensitivity of SCAR marker. The specificity of selected primer pair was further verified by PCR with genomic DNA of 15 isolates of *C. globosum*, and one isolate each of *C. reflexum*, *C. cochlioides*, *C. perlucidum* and *C. cupreum.* Additionally, 10 isolates of other fungal plant pathogens collected from diverse locations, not used for primer design and PCR assay were included for validation (Table 1). A water blank as negative control and

C. globosum DNA as positive control were included for PCR amplification. In order to test the sensitivity of SCAR marker, 100 ng, 10 ng, 1 ng, 500 pg, 250 pg, 100 pg, 75 pg and 50 pg genomic DNA of *C. globosum* (Cg2) were used as DNA template for PCR amplification.

PCR-based detection of *C. globosum* in wheat leaves, root and rhizosphere in the presence of pathogen. This study was conducted for verifying the detection potential of the designed primers in *C. globosum*-inoculated soil and plant separately and in the presence of *B. sorokiniana*, a spot blotch pathogen of wheat. For inoculation purpose, *C. globosum* and *B. sorokiniana* multiplied on PDA were used. Small pots (4 inches) were filled with sterilized soil and divided into four sets, each with six replicates. Seeds were surface-disinfected with 2% NaOCl for 3 min and rinsed thrice in sterile distilled water before sowing in all set of pots. In one set, soil was amended with *C. globosum* ascospore suspension at 10^8 CFU/g and in second set soil was amended with both *C. globosum* ascospore suspension and *B. sorokiniana* spores at 10^8 CFU/g each. In a third set, wheat plants were sprayed with ascospores of *C. globosum* at 10^8 CFU/ml at 2-3 leaf stage (growth stage Z-13) (Zadoks *et al*., 1974) using a hand atomizer, while a fourth unsprayed set was maintained as control.

Root and soil samples from the first two sets and leaves samples from the third set were colleted to detect *C. globosum* after 4 days of application. Samples were also taken from the fourth set for control. DNA was extracted from different samples using a ZR soil microbe DNA extraction kit. The presence of *C. globosum* was detected by PCR in suitable conditions standardized earlier.

Real-time PCR assays. *Design of qPCR primers*: A primer set for qPCR was designed from SCAR marker using the Primer Express software program version 2.0 (Applied Biosystems, USA). An *in silico* test for primer specificity was conducted by running the primer sequence against the non redundant GenBank data with parameters set for the identification of short, nearly exact matches.

Optimization of qPCR conditions: PCR conditions, i.e annealing temperature, primer concentration, temperature to measure the fluorescence signal of specific amplicon using *C. globosum* DNA as template were adjusted experimentally to optimize the qPCR. All qPCR amplifications were taken up using Thermo Scientific Maxima SYBR Green qPCR Master Mix, iQ96-thin well PCR plates and optical sealing tape (Roche 96, USA). Light Cycler (Roche 96, USA) conditions were as follows: 95°C for 10 min, and 40 cycles of PCR amplification at 95°C for 15 sec and 62°C for 30 sec followed by default melt curve analysis. Sterile water was used as a negative control to replace template DNA in PCR reactions. All reactions were analysed by gel electrophoresis to confirm that only one PCR product was amplified from samples containing genomic DNA of *C. globosum*. Ct values were calculated by the ABI Prism 7000 SDS software program (Applied Biosystems, USA) to indicate significant fluorescence signals rising above background during the early cycles of the exponentially growing phase of the PCR amplification process.

Development of standard curve: DNA standard curve for qPCR assay were developed from 10 fold dilution of *C. globosum* isolate Cg2 DNA by serial dilution (100 fg, 1 pg, 10 pg, 100 pg, 1 ng and 10 ng) in sterile ultrapure water (SUW) as well as in fixed background of plant DNA extracted from leaf, root and sterilized soil. The standard curve was obtained by plotting the Ct values defined by the crossing cycle number versus the logarithm of the quantity of the serially diluted genomic DNA. Linear regression analyses of the natural logarithm base 10 of known concentrations of target DNA verses Ct values were performed for each standard curve. The standard regression lines from different DNA back ground were used as reference curve for transforming the experimental Ct values into amount of *C. globosum* DNA (pg).

Quantification of C. globosum target DNA in soil using qPCR: Soil samples were collected from rhizosphere of wheat plants at 0,7 days and 15 days time intervals from the first two sets of pots as mentioned earlier for detecting and quantifying DNA concentration using optimized qPCR and standard curve. For quantification of target DNA from soil samples, two µl of undiluted DNA extracted from different soil samples were included as template in the real-time PCR reactions. Concentration of the target DNA was estimated by interpolating the cycle threshold (Ct values) of sample DNA obtained from qPCR with the standard curve. The concentration so obtained was further calculated for target DNA per g soil. All reactions were carried out in triplicate.

RESULTS

Development of specific and sensitive SCAR marker. Out of 12 URP primers tested, primers URP-2R, URP-6R and URP-7R produced monomorphic bands of different size from all *C. globosum* isolates tested. Out of these selected primers, URP-2R (CCCAGCAACTGATCGACAC) amplified a single band of 1.9 kb only in *C. globosum* isolates (Fig. 1). This amplicon was cloned into pGEM-T easy vector and out of 20 white colonies picked up, five clones were sequenced. All the clones showed identical sequences. Nucleotide sequence alignment, done using the BioEdit program confirmed the URP fragment with the primer sequence (URP-2R) at both ends. No ORF could be detected in this fragment. Fragment showed 77%, 54% and 55% homology with three accessions of *C. globosum* i.e. XM001225295.1, XM001224275.1 and XM001224636.1, consisting of three conserved domains [putative DNA

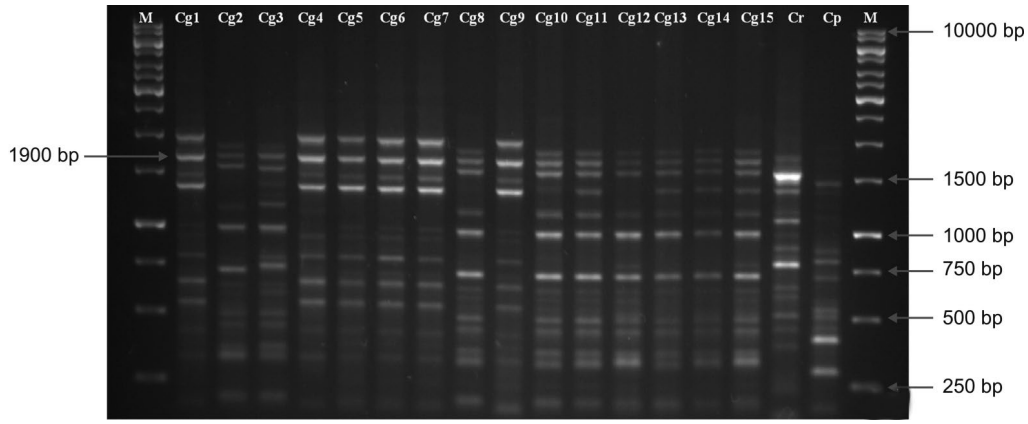

Fig. 1. DNA fingerprint profile of different isolates of *Chaetomium* spp. obtained with primer URP-2R. M-1Kb Molecular marker, Fermentas; (lanes 1-15) *C. globosum* isolates; (Cr) *C.reflexum*; (Cp) *C. perlucidum*; M-1Kb Molecular marker, Fermentas.

Fig. 2. Agarose gel showing specificity of primer set SCCgRAF4/ SCCgRAR4 to *C. globosum.* M-1Kb Molecular marker, Fermentas; (1) *C. globosum*; (2) *C. perlucidum*; (3) *C. reflexum*; (4) *C. cupreum*; (5) *C. cochlioides*; (6) *Bipolaris sorokiniana*; (7) *Fusarium moniliforme*; (8) *Alternaria triticina*; (9) *Tilletia indica***;** (10) *Epiccocum purpurecens*; (11) *Puccinia striiformis*; (12) *Trichoderma virens*; wheat leaf (13) DNA; (14) distilled water.

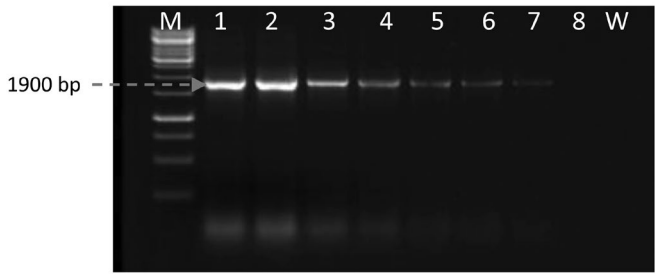

Fig. 3. Sensitivity of conventional PCR assays detected by agarose gel electrophoresis using SCCgRAF4/ SCCgRAR4 primer set. M-1Kb Molecular marker, Fermentas; (1) 100 ng; (2) 10 ng; (3) 1 ng; (4) 500 pg; (5) 250 pg; (6) 100 pg; (7) 75 pg; (8) 50 pg; (W) sterile water.

Table 2. Primers designed from the cloned sequence (accession No. KC200158)

Serial No.	Name of Primer	Nucleotide sequence
1	SCCgRAF1/SCCgRAR1	5'-TGG CGT AAC ACT CCC ACA TA-3'/ 5' - TCC AAG GTC AAA CCA CAA CA-3'
2	SCCgRAF2/SCCgRAR2	5'-ACT GAT CGC ACA CAC CAC CAA T-3' / 5'- ACT GAT CGC ACA CTC CAC CTCT-3'
3	SCCgRAF3/SCCgRAR3	5'-ACA CAC CAC CAA TCG CAC ACTT-3' / 5'- ACT GAT CGC ACA CTC CAC CTCT-3'
4	SCCgRAF4/SCCgRAR4	5'-CAC CAA TCG CAC ACT TTG ACC-3' / 5'- ACT GAT CGC ACA CTC CAC CTCT-3'

binding domain (smart00674), Rve integrase core domain (C101316) and Psq domain (Pfam 05225)], otherwise there was no sequence homology with any other existing nucleotide and protein sequences in databases. After removing the vector sequence, a sequence of 1,900 bp specific to *C. globosum* was left. Based on this sequence (accession No. KC200158), out of four primer pairs (Table 2), the primer set SCCgRAF4 (5'- CAC CAA TCG CAC ACT TTG ACC-3')/SCCgRAR4 (5'- ACT GAT CGC ACA CTC CAC CTCT-3') amplified the desired DNA band of 1,900 bp (SCCg RA_{1900}) from all 15 isolates of *C. globosum* but not *C. reflexum* and *C. perlucidum* (data not shown). The optimal conditions for PCR were found to be: one cycle of denaturation at 94°C for 4 min followed by 40 cycles of denaturation at 94°C for 40 sec, annealing at 65°C for 30 sec and extension at 72°C for 60 sec and a final extension at 72°C for 7 min. The SCAR marker (SCCg RA_{1900}) displayed high specificity and clearly distinguished *C. globosum* from other *Chaetomium* species and other fungal isolates by producing an amplicon of 1.9 kb (Fig. 2). The sensitivity of the marker using a dilution series of total genomic DNA extracted from *C. globosum* revealed that as little as 75 pg template was sufficient for conventional PCR-based diagnostics (Fig. 3).

PCR-based detection of *C. globosum* at the place of application. The universality of the marker was tested to detect the presence of the biocontrol agent on wheat leaves, root and soil. The predicted amplified product of 1.9 kb was obtained with PCR amplification of DNA from wheat leaves 4 days post application with a *C. globosum* bioformulation. Leaves from untreated plants did not show any amplification. A clear band was also amplified with DNA from *C. globosum*-amended soil samples and roots.

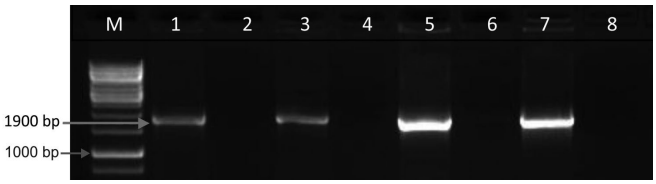

Fig. 4. Representative agarose gel showing amplification products from polymerase chain reaction using SCCgRAF4/SCCgRAR4 primer set: M-1Kb, Molecular marker; (1) DNA from leaf sprayed with *C. globosum*; (2) DNA from *C. globosum* unsprayed samples; (3) DNA from root sample amended with *C. globosum*; (4) DNA from root without *C. globosum* amendment; (5) DNA from soil amended with *C. globosum*; (6) soil without *C. globosum*; (7) DNA from pure culture of *C. globosum* ; (8) Water, negative control.

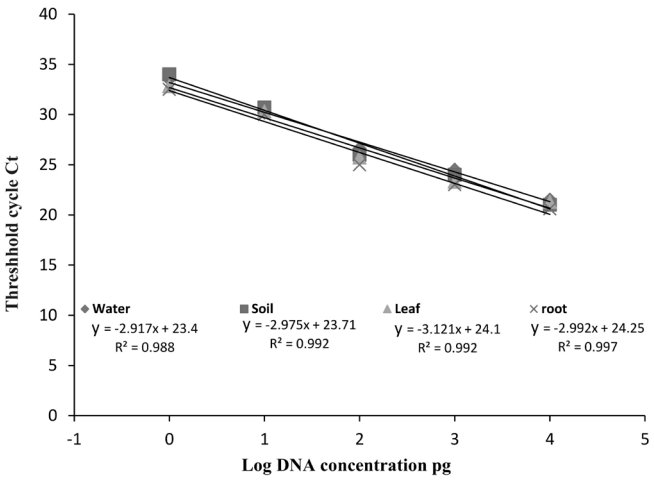

Fig. 5. Standard curve showing the $\log_{10}$ DNA amount (pg) plotted against the realtime PCR cycle threshold (Ct) for different dilutions of pure genomic DNA of *Chaetomium globosum* in sterile ultrapure water (SUW) as well as in fixed background of uninoculated leaf, root and from sterilized soil. R^2 indicates a linear relationship between the DNA amount and Ct values.

No PCR amplification was observed from DNA of uninoculated soil, uninoculated plants and negative control (sterile water). (Fig. 4).

Real time PCR-based quantification of *C. globosum*. A primer set SCCgQF (5'-GCGTCGGGACAGTCTTATTTG-3') and SCCgQR (5'-GCCATCGGTCACAAGGAAGT-3') was designed using SCAR marker ($SCCgRA_{1900}$) for qPCR. PCR mix consisting of 2 µl undiluted DNA template, 10 µl PCR master mix with SYBR Green Thermo Scientific Maxima SYBR Green qPCR Master Mix, 1 µl (10 µM) of each primer and 6 µl of nuclease-free water found optimal for qPCR. Target DNA showed fluorescence but no fluorescence signals occurred for negative controls. The standard curve drawn using input *C. globosum* DNA in SUW and in fixed background of soil, leaf and root DNA showed a linear correlation between Ct value and DNA concentration (pg), with a correlation coefficient of 0.988, 0.992, 0.992 and 0.997 respectively, showing the accuracy of real time PCR-based quantification. Homogeneity of standard curves showed that background DNA did not affect the result of qPCR assay. First fluorescent signals were observed at Ct 33.5 corresponding approximately to 1 pg DNA, while at Ct 20 it reached to highest concentration (10 ng) (Fig. 5). Amplification was confirmed by the melting curve that showed one distinct peak (Fig. 6) and agarose gel analysis showing presence of a distinct band of expected size 63 bp (data not shown). Soil sample analysis revealed that unamended soil did not show any fluorescence, however DNA from soil amended with *C. globosum* showed significant fluorescence. Initial Ct value of real time PCR in *C.globosum*-amended soil was 28.2, equivalent to 0.50 ng target DNA/g soil, which increased 6 times (3.10 ng/g soil; Ct = 25.78) after 7 days of amendment and reached to 4.07 ng/g after

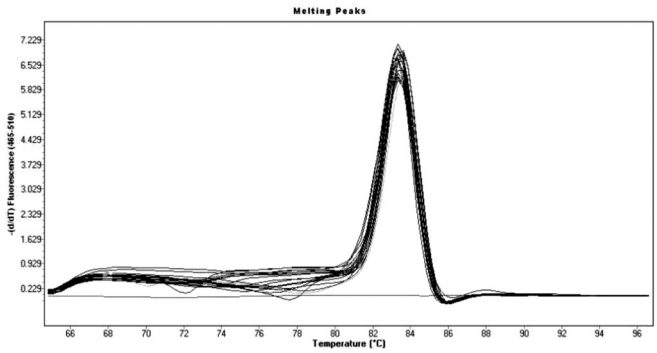

Fig. 6. Melting curve of qPCR products obtained using *Chaetomium globosum* specific primers SCCgQF and SCCgQR, no peak was observed in negative control.

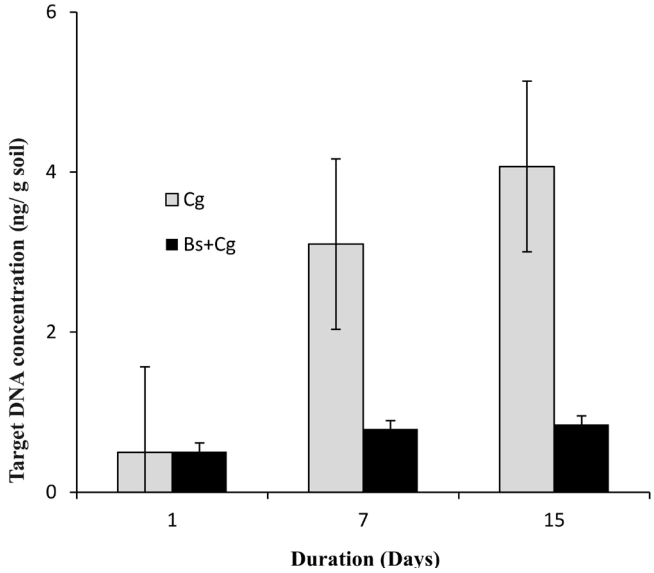

Fig. 7. DNA concentration of *Chaetomium globosum* estimated by qPCR at different durations in only *C. globosum* amended soil and in soil amended with both *C. globosum* and *Bipolaris sorokiniana* (Bs+Cg). Error bars represent the standard error of the mean M.

15 days (Ct = 25.5). In another experiment where *C. globosum* was co-amended with pathogen, *B. sorokiniana*, initial target DNA quantity of *C. globosum* was 0.51 ng/g soil (Ct = 28.1) which showed increase (0.88 ng/g soil) in 15 days (Ct = 27.4) (Fig. 7).

DISCUSSION

C. globosum is an emerging BCA against many fungal plant diseases. It has been identified as a potential BCA against a number of soil-borne and foliar pathogens (Di Pietro *et al*., 1992; Soytong and Quimio, 1989). Our studies have also shown its bioefficacy against spot blotch disease of wheat (Aggarwal *et al*., 2004) and *Ascochyta* blight of chick pea (Rajkumar *et al*., 2005). It is important to monitor the persistence and release of BCAs into the environment accurately. PCR methods for genus/species and strain identification of microorganisms are developed to minimize the time required for classical microbiological and enzymatic methods (Sanzani *et al*., 2012). The identification of fungal species/strain on the basis of their DNA requires the characterization of discriminating DNA targets. During this study, a distinct marker has been developed for detection of *C. globosum* which can differentiate this fungus from isolates of different species of *Chaetomium* and other soil fungi. This marker was also able to detect *C. globosum* at the site of application in wheat tissues and soil samples.

Conserved genome regions such as the ITS sequences of rDNA and mtDNA have been chosen for taxonomic purposes at the species level in most fungi (Mbofung and Pryor, 2010; McKay *et al*., 1999; Miyazaki *et al*., 2009). Other conserved genes, such as β-tubulin (Hirsch *et al*., 2000), and elongation factors (Filion *et al*., 2003; Li and Hartman, 2003) have also been used as specific markers. However, in studies conducted in our laboratory on ITS region, β-tubulin, GPD and the gene xylanase, it was observed that all the tested isolates of *Chaetomium* spp. showed approximately 90% sequence homology with each other. This indistinguishability among different species of *Chaetomium* makes it difficult to develop a specific marker. RAPD/URP technique has been found to be more useful for detecting genomic polymorphism as this generates neutral markers that may reflect the whole genotype of an individual. Earlier, Aggarwal *et al.* (2008) had carried out the molecular characterization of *C. globosum* using URP-primers. This work highlights the development of SCAR marker from a unique region of genomic DNA of *C. globosum* identified after wide screening of different species of *Chaetomium* and other pathogens using 12 URP primers. Primer, URP-2R amplified an unique 1900 bp band only from *C. globosum*. Species-specific primers were designed from this band. Careful design and selection of primers having their homology with the target molecule and lack of homology from taxonomically diverse fungal species can significantly improve the sensitivity of PCR-based markers (He *et al*., 1994). Primer pair SCCgRAF4/ SCCgRAR4 designed from this band amplified a distinct band of 1,900 bp from genomic DNA of all isolates of C. *globosum*, but not from DNA of closely related species of *Chaetomium* or other fungi. This specific sequence when blasted in NCBI data base was shown to contain a transposase domain, which normally creates variability by either insertion or deletion (Grzebellus *et al*., 2007). Therefore, they are suitable for developing specific markers.

$SCCgRA_{1900}$ marker required only a few hours for detection of the BCA at the place of application. However, the amount of *C. globosum* DNA could not be accurately quantified with conventional PCR because the efficiency of DNA amplification is variable between reactions (Raeymaekers, 1998). To overcome this problem qPCR-based detection was developed which allows BCA quantification in soil and in different plant parts accurately. Moreover, the sensitivity of qPCR assay was not affected by co-extraction of host DNA as we found during conventional PCR for BCA detection from soil and plant. A primer pair SCCgQF and SCCgQR designed from SCAR marker which amplified a 63 bp product could detect as much as 1 pg of DNA (4.83×10^5 copy number of target DNA) of *C. globosum* in soil/plant tissues using qPCR.

Real time PCR has widely been uses for detection of BCAs and fungal pathogens in soil (Ruben *et al*., 2009; Beaulieu *et al*., 2010; Savazzini *et al*., 2008; Sanzani *et al*., 2012, 2014), but this is the first example of its application for quantifying *C. globosum*. Currently available techniques for *C. globosum* quantification utilize soil dilution plating. The identification of *C. globosum* from these plates at the early stage of growth is tedious and requires detailed microscopic observations, which is time consuming. qPCR protocol developed here can accurately identify and quantify *C. globosum* population in terms of DNA concentration. Our result indicated that initial Ct value of soil sample from *C. globosum*-amended soil was 28.1 which was equivalent to 2.5×10^8 copy number/g soil (0.51 ng target DNA/g soil sample). After seven days this increased to 25.74, equivalent to 1.52×10^9 copy number/g soil (3.1 ng./g soil) indicating approximately a 9 fold increase in population in terms of copy number. Further, in 15 days Ct reached up to 25.5, showing a *C. globosum* population of 2×10^9 copy number/g soil (4.07 ng target DNA /g soil). These results suggest that *C. globosum* can be a successful BCA since its population increases over time in soil.

Earlier workers have also reported the colonization of microorganisms in soil and their spread at the site of application that can successfully be monitored over time and space (Van Veen *et al*., 1997; Whipps, 2001; Alabouvette *et al*., 2006). The colonization by BCA is also influenced by the virulence of prevailing pathogens in soil or plants. Also in such conditions qPCR can quantify *C. globosum* in the soil and performs as an useful tool for studying its population. In the present investigation, qPCR quantified *C. globosum* target DNA concentration in the presence of

B. sorokiniana, which was initially 0.51 ng/g soil (2.5×10^8 copy number of target DNA) and increased slowly to reach to 0.88 ng/g soil (4.32×10^8 copy number) after 15 days of amendment. This may indicate that *C. globosum* interacts with this pathogen. Our study could be further utilized for emphasizing the competitive saprophytic ability of *C. globosum*, against various soil-borne pathogens.

In conclusion, this is the first report of successful detection and quantification of *C. globosum*, from soil using qPCR technique, although there are reports of detection and quantification of *Trichoderma* species (Cordier *et al*., 2007; Rubio *et al*., 2005). Thus, we have developed a highly sensitive, reliable, rapid, reproducible method for detection of this BCA at its place of application. The qPCR-based assay can be used for monitoring *C. globosum* population and help in applying the appropriate doses of the bioformulation at the appropriate time to restrict the growth of pathogens. This DNA-based technology is faster than time-consuming and labour-intensive traditional culturing methods. Moreover, the molecular approach can analyze large number of samples and will facilitate the tracking of BCAs in natural environments.

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REFERENCES

Aggarwal R., Tiwari A.K., Srivastava K.D., Singh D.V., 2004. Role of antibiosis in the biological control of spot blotch (*Cochliobolus sativus*) of wheat by *Chaetomium globosum*. *Mycopathologia* **15**: 369-377.

Aggarwal R., Sharma V., Kharbikar L., Renu, 2008. Molecular characterization of *Chaetomium* species using URP-PCR. *Genetics and Molecular Biology* **31**: 943-946.

Aggarwal R., Singh V.B., Shukla R., Gurjar M.S., Gupta S., Sharma T.R., 2010. URP-based DNA fingerprinting of *Bipolaris sorokiniana* isolates causing spot blotch of wheat. *Journal of Phytopathology* **158**: 210-216.

Aggarwal R., Gupta S., Banerjee S., Singh V.B., 2012. Development of SCAR marker for detection of *Bipolaris sorokiniana* causing spot blotch of wheat. *Canadian Journal of Microbiology* **57**: 934-942.

Ahammed S.K., Aggarwal R., Kapoor H.C., 2008. Production, partial purification and characterization of extracellular xylanase from *Chaetomium globosum*. *Journal of Plant Biochemistry Biotechnology* **17**: 95-98.

Ahammed S.K., Aggarwal R., Sharma S., Gupta S., Bashyal B.M., 2012. Production, partial purification and characterization of extra-cellular β-1, 3-glucanase from *Chaetomium globosum* and its antifungal activity against *Bipolaris sorokiniana* causing spot blotch of wheat. *Journal of Mycology and Plant Pathology* **42**: 146-152.

Alabouvette C., Olivain C., Steinberg C., 2006. Biological control of plant diseases: the European situation. *European Journal of Plant Pathology* **114**: 329-341.

Beaulieu R, López-Mondéjar. R., Tittarelli F., Ros M., Pascual J.A., 2010. qRT-PCR quantification of the biological control agent *Trichoderma harzianum* in peat and compost-based growing media. *Bioresourse Technology* **102**: 2793-2798.

Becker E., M Ball L.A., Hintz W.E., 1999. PCR-based genetic markers for detection and infection frequency analysis of the biocontrol fungus *Chondostereum purpureum* on Sitka alder and trembling aspen. *Biological Control* **15**: 71-80.

Biswas S.K., Aggarwal R., Srivastava K.D., Gupta S., Dureja P., 2012. Characterization of antifungal metabolites of C*haetomium globosum* Kunze and their antagonism against fungal plant pathogens. *Journal of Biological Control* **26**: 70-74.

Bulat S.A., Lübeck M., Mironenko N., Jensen D.F., Lübeck P.S., 1998. UP-PCR analysis and ITS1 ribotyping of strains of *Trichoderma* and *Gliocladium*. *Mycological Research* **102**: 933-943.

Cordier C., Edel-Hermann V., Martin-Laurent F., Blal B., Steinberg C., Alabouvette C., 2007. SCAR -based real time PCR to identify a biocontrol strain (T1) of *Trichoderma atroviride* and study its population dynamics in soils. *Journal of Microbiological Methods* **68**: 60-68.

Di Pietro A., Gut-Rella M., Pachlatko J.P., Schwinn F.J., 1992. Role of antibiotics produced by *Chaetomium globosum* in biocontrol of *Pythium ultimum*, a causal agent of damping-off. *Phytopathology* **82**: 131-135.

Filion M., St-Arnaud M., Jabaji-Hare S.H., 2003. Direct quantification of fungal DNA from soil substrate using real-time PCR. *Journal of Microbiological Methods* **53**: 67-76.

Grzebellus D., Jagosz B., Simon P., 2007. The Dc Master transposon disply maps polymorphic insertion sites in carrot (*Daucus carota* L.) genome. *Gene* **390**: 67-74.

Harman G.E., Eckenrode C.J., Webb D.R., 1978. Alteration of spermosphere ecosystems affecting oviposition by the bean seed fly and attack by soil borne fungi on germinating seeds. *Annals of Applied Biology* **90**: 1-6.

He Q**.,** Marjamaki M., Soini H., Mertsola J., Viljanen M.K., 1994. Primers are decisive for sensitivity of PCR. *Biotechniques* **17**: 82-87.

Hirsch P.R., Mauchline T.H., Mendum T.A., Kerry B.R., 2000. Detection of the nematophagous fungus *Verticillium chlamydosporium* in nematode-infested plant roots using PCR. *Mycological Research* **104**: 435-439.

Li S., Hartman G.L., 2003. Molecular detection of *Fusarium solani* f. sp. *glycines* in soybean roots and soil. *Plant Pathology* **52**: 74-83.

Lo C.T., Nelson E.B., Harman G.E., 1996. Biological control of turf grass diseases with a rhizosphere component strain of *Trichoderma harzianum*. *Plant Disease* **82**: 736-741.

Lübeck M., Alekhina I.A., Lübeck P.S., Jensen D.F., Bulat S.A., 1999. Delineation of *Trichoderma harzianum* into two different genotypic groups by a highly robust fingerprinting method, UP-PCR, and UP-PCR product cross-hybridization. *Mycological Research* **103**: 289-298.

Mavrodi O.V., Mavrodi D.V., Thomashow L.S., Weller D.M., 2007. Quantification of 2, 4-diacetylphloroglucinol-producing *Pseudomonas fluorescens* strains in the plant rhizosphere by real-time PCR. *Applied Environmental Microbiology* **73**: 5531-5538.

Mbofung G.C.Y., Pryor B.M., 2010. A PCR-based assay for detection of *Fusarium oxysporum* f. sp. *lactucae* in lettuce seed. *Plant Disease* **94**: 860-866.

McKay G.J., Brow A.E., Bjourson A.J., Mercer P.C., 1999. Molecular characterization of *Alternaria linicola* and its detection in linseed. *European Journal of Plant Pathology* **105**: 157-166.

Miyazaki K., Tsuchiya Y., Okuda T., 2009. Specific PCR assays for the detection of *Trichoderma harzianum* causing green mold disease during mushroom cultivation. *Mycoscience* **50**: 94-99.

Murray M.G., Thompson W.F., 1980. Rapid isolation of high molecular weight plant DNA. *Nucleic Acid Research* **8**: 4321-4326.

Raeymaekers L., 1998. Quantitative PCR. In: Lo Y.M.D. (ed). Methods in Molecular Medicine: Clinical application of PCR,. Humana Press, Totowa, NJ, USA **16**: 27-38.

Rajkumar E., Aggarwal R., Singh B., 2005. Fungal antagonists for the biological control of Ascochyta blight of chickpea. *Acta Phytopathologica et Entomologica Hungarica* **40**: 35-42.

Rubén López-Mondéjar Anabel A., Stefan R., Margarita R., José A.P., 2009. Quantification of the biocontrol agent *Trichoderma harzianum* with real-time TaqMan PCR and its potential extrapolation to the hyphal biomass. *Bioresource Technology* **101**: 2888-2891.

Rubio M.B., Hermosa M.R., Keck E., Monte E., 2005. Specific PCR assays for the detection and quantification of DNA from the biocontrol strain *Trichoderma harzianum* 2413 in soil. *Microbial Ecology* **49**: 25-33.

Sambrook J., Russell D.W., 2001. Molecular Cloning: A Laboratory Manual, Vol. 3, Cold Spring Harbour Laboratory Press, New York, USA.

Sanzani S.M., Schena L., De Cicco V., Ippolito A., 2012. Early detection of *Botrytis cinerea* latent infections as a tool to improve postharvest quality of table grapes. *Postharvest Biology and Technology* **68**: 64-71.

Sanzani S.M., Li Destri Nicosia M.G., Faedda R., Cacciola S.O., Schena L., 2014. Use of quantitative PCR detection methods to study biocontrol agents and phytopathogenic fungi and oomycetes in environmental samples. *Journal of Phytopathology* **162**: 1-13

Savazzini F., Longa C.M.O., Pertot I., Gessler C., 2008. Real-time PCR for detection and quantification of the biocontrol agent *Trichoderma atroviride* strain SC1 in soil. *Journal of Microbiological Methods* **73**: 185-194.

Schena L., Nigro F., Ippolito A., 2002 . Identification and detection of *Rosellinia necatrix* by conventional and real-time PCR. *European Journal of Plant Pathology* **108**: 355-366.

Schena L., Li Destri Nicosia M.G., Sanzani S.M., Faedda R., Ippolito A., Cacciola S.O., 2013. Development of quantitative PCR detection methods for phytopathogenic fungi and oomycetes. *Journal of Plant Pathology* **85**: 15-25.

Soytong K., Quimio T.H., 1989. Antagonism of *Chaetomium globosum* to rice blast pathogen *Pyricularia oryzae*. *Kasetsart Journal*, *Natural Science* **23**: 198-203.

Suarez M.B., Walsh K., Boonham N., O'Neill T., Pearson, S., Barker I., 2005. Development of real-time PCR (TaqMan) assays for the detection and quantification of *Botrytis cinerea in planta*. *Plant Physiology and Biotechnology* **43**: 890-899.

Tyler J.A., Richard C., Richard H., Bélanger R., 2001. Approaches to molecular characterization of fungal biocontrol agents: some case studies. *Canadian Journal of Plant Pathology* **23**: 8-12.

Van Veen J.A., Van Overbeek L.S., Van Elsas, J.D., 1997. Fate and activity of microorganisms introduced into soil. *Microbiology and Molecular Biology Reviews* **61**: 121-135.

Vannacci G., Harman G.F., 1987. Biocontrol of seed borne *Alternaria raphani* and *A. brassicicola*. *Canadian Journal of Microbiological Research* **33**: 850-856.

Whipps J.M., 2001. Microbial interactions and biocontrol in the rhizosphere. *Journal of Experimental Botany* **52**: 487-512.

Xiang S.R., Cook M., Saucier S., Gillespie P., Socha, R., Scroggins R., Beaudette L.A., 2010. Development of amplified fragment length polymorphism-derived functional strain-specific markers to assess the persistence of 10 bacterial strains in soil microcosms. *Applied Environmental Microbiology* **76**: 7126-7135.

Zadoks J.C., Chang T.T., Konzak C.F., 1974. Decimal code for growth stages of cereals. *Weed Research* **14**: 415-421.

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