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Strain-specific detection of introduced *Beauveria bassiana* in agricultural fields by use of sequence-characterized amplified region markers

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Abstract

Field studies on the efficacy and persistence of an introduced strain of *Beauveria bassiana* for insect control require detection assays to differentiate the non-native strain from indigenous populations. In this study we developed strain-specific molecular markers based on polymerase chain reaction amplification of sequence-characterized amplified regions (SCAR) in combination with dilution plating on semi-selective medium to detect and estimate density of propagules of a commercial strain of *B. bassiana* (strain GHA) in field samples. Using random amplified polymorphic DNA (RAPD) analysis, unique fragments that distinguished GHA from other strains of *B. bassiana* were obtained. Three amplicons, OPA-$14_{0.44}$, OPA-$15_{0.44}$, and OPB-$9_{0.67}$, generated with RAPD primers were cloned and sequenced and used as bases for designing SCAR primers OPA14 F/R_{445}, OPA15 F/R_{441}, and OPB9 F/R_{677}, respectively. All three SCAR primers were highly sensitive, capable of detecting 100 pg *B. bassiana* GHA genomic DNA, and thus could be used to detect varying levels of the fungus in the field.
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Keywords: *Beauveria bassiana*; Entomopathogenic fungus; Microbial control; Molecular markers; Sequence-characterized amplified region markers; Strain detection

1. Introduction

Field studies on the efficacy and persistence of an introduced microbial control agent require detection assays to differentiate the non-native strain from indigenous populations. Such techniques are critical for the entomopathogenic fungus *Beauveria bassiana* because this fungus has a wide insect host range and is common in nature (McCoy et al., 1988). There are also several *B. bassiana*-based mycoinsecticides currently registered or under commercial development worldwide for agricultural pests (Hajek et al., 2001). Field releases of these mycoinsecticides add to fungal populations in the field and may become established over time. The presence of indigenous populations that can produce an outbreak in the target pest population and mask or enhance the effects of an introduced fungal strain makes it necessary for surveys of *B. bassiana* to be conducted prior to and after field applications. These surveys identify the etiological agent(s) attacking target insects and verify the impact of the introduced fungal strain on insect pest populations.

Molecular markers have been utilized to assess genetic variation among isolates of *B. bassiana* and other entomopathogenic fungi, thereby providing means to identify strains of interest, determine origin of isolates, or study population structure. One technique that has been used to differentiate strains of *B. bassiana* is polymerase chain reaction (PCR)-based random amplified polymorphic DNA (RAPD) (Bidochka et al., 1994; Castrillo et al., 1999; Maurer et al., 1997). This technique utilizes short primers of arbitrary sequence that anneal to multiple target sequences producing diagnostic patterns (Williams et al., 1990). Because RAPD analysis does not require prior knowledge of target site sequence, it can be easily adapted to study various entomogenous fungi, even those with poorly studied genomes.

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RAPD analysis has also been utilized to generate unique PCR products or amplicons in filamentous fungal species or strains of interest to be converted into species- or strain-specific sequence-characterized amplified region (SCAR) markers (Abbasi et al., 1999; Lecomte et al., 2000; Li et al., 1999; Schilling et al., 1996). SCAR markers differ from RAPD markers in that SCAR primers are designed based on known DNA sequences of the organism in study. This allows for the development of sensitive and diagnostic assays to amplify specific fungal DNA in field samples containing mixed DNA because primers anneal specifically to fungal sequences. This is in contrast to RAPD analysis that requires the establishment of single spore isolates for strain identification.

We are currently evaluating *B. bassiana* strain GHA as a microbial control agent against the Colorado potato beetle and other agricultural insect pests. In this study we utilized RAPD-PCR technique to screen for markers that would differentiate *B. bassiana* GHA from other strains of the fungus. These RAPD markers were converted to SCAR markers for development of a sensitive diagnostic assay for the selective detection of *B. bassiana* GHA. Additionally, we combined SCAR assays with dilution plating to estimate density of fungal propagules present in foliage and soil samples from field plots.

2. Materials and methods

2.1. Fungal isolates and DNA extraction

Strains of *B. bassiana*, *Beauveria amorpha*, *Beauveria brongniartii*, and *Metarhizium anisopliae* were obtained from the USDA-ARS Entomopathogenic Fungi (ARSEF) Culture Collection in Ithaca, NY, USA. *B. bassiana* strain GHA, the active ingredient in commercial products registered for control of several agricultural insect pests, was isolated from a technical product (Lot No. 980528) provided by Mycotech (now Emerald Bio-Agriculture, Lansing, MI). Strains of *B. bassiana* isolated from infected Colorado potato beetles collected in July 1997 and in July 2000 from non-GHA-treated potato fields in Freeville, NY, were included in the study to represent naturally occurring strains at the test site. Information on insect host, collection site and year of isolation for each fungal strain are listed in Table 1. Single spore isolates were established for each strain and were cultured on Sabouraud dextrose agar supplemented with 0.1% yeast extract (SDAY) for 10 days at room temperature (~24 °C). Cultures were maintained on SDAY plates stored at 4 °C or agar blocks ($0.5 \times 1\,cm^2$) cut from growing cultures were stored in cryogenic vials with 10% glycerol at −80 °C (Humber, 1997).

Fungal genomic DNA for screening RAPD and SCAR markers was isolated from blastospores grown in liquid medium following the method of Pfeifer and Khachatourians (1993) with modification: 100 mg *Penicillium funiculosum* cellulase and 100 mg *Trichoderma viridae* cellulase were replaced with 100 mg *Aspergillus niger* cellulase and 50 mg *Trichoderma harzianum* cellulase (Sigma), respectively. DNA concentration was determined by use of a spectrophotometer (Pharmacia Biotech) or by running aliquots of DNA extracts against a molecular mass ladder (Life Technologies).

2.2. RAPD analysis and PCR conditions

A preliminary screening was conducted using nine *B. bassiana* strains, including strain GHA, against 10-nucleotide random primers obtained from Operon Technologies. A total of 88 RAPD primers were screened, from which four primers producing robust, reproducible, and unique amplicons of less than 1 kb in *B. bassiana* GHA were selected. The four primers selected were OPA-5 (5′-AGGGGTCTTG), OPA-14 (5′-TCTGTGC TGG), OPA-15 (5′-TTCCGAACCC), and OPB-9 (5′-TGGGGGACTC). These primers were screened against 43 strains of *B. bassiana* and seven strains of *B. amorpha*, *B. brongniartii*, and *M. anisopliae* (Table 1). Four *B. bassiana* GHA isolates were included in the assays to test the homogeneity of their source and to ensure consistency of amplicons generated. PCR mixtures (25 μl volume) contained 1× PCR buffer with 1.5 mM $MgCl_2$ (Qiagen); 200 μM each of dATP, dCTP, dGTP, and dTTP (Qiagen); 0.5 μM RAPD primer; 10 ng fungal DNA; and 2.5 U *Taq* PCR enzyme (Qiagen). PCR amplification was performed in a PTC-200 thermal cycler (MJ Research) programmed for initial denaturation at 94 °C for 4 min; 35 cycles of denaturation at 94 °C for 60 s, annealing at 37 °C for 60 s, and extension at 72 °C for 2 min; and a final extension step at 72 °C for 5 min. Reaction tubes were held at 4 °C prior to visualization of PCR products in a 1.0% agarose gel stained with ethidium bromide. Each RAPD assay was done three times to ensure reproducibility.

2.3. Cloning and sequencing of strain-specific RAPD amplicons

The selected diagnostic RAPD marker for *B. bassiana* GHA from each of the four primers was excised from agarose gels and the DNA fragment was recovered using QIAquick gel extraction kit (Qiagen) following the manufacturer's protocol. An aliquot of the recovered DNA fragment was reamplified using the corresponding primer to verify that only a single band was excised. The RAPD markers were cloned using a Perfectly Blunt Cloning kit with pSTBlue-1 vector (Novagen). Then, NovaBlue competent cells (Novagen) were transformed with ligated DNA following the manufacturer's instructions. Plasmid DNA from recombinants was

Table 1
Strains of *B. bassiana* and other entomopathogenic fungi investigated in this study

Species and strains	Host origin	Collection site	Year
Beauveria sp.			
ARSEF 32	(Orthoptera: Acrididae)	Montana	1973
B. bassiana			
GHA	From technical product (Mycotech Industries)		2000
ARSEF 1	*Malacosoma americanum* (Lepidoptera: Lasiocampidae)	Dennis, Massachusetts	1972
ARSEF 201	*Diabrotica undecimpunctata* (Coleoptera: Chrysomelidae)	Corvalis, Oregon	1977
ARSEF 252	*Leptinotarsa decemlineta* (Coleoptera: Chrysomelidae)	Orono, Maine	1978
ARSEF 292	Reisolate of Boverin	Commonwealth of Independent States	1978
ARSEF 317	*L. decemlineata* (Coleoptera: Chrysomelidae)	Rhode Island	1979
ARSEF 318	(Coleoptera: Cerambycidae)	Rhode Island	1979
ARSEF 350	Not specified		
ARSEF 353	*L. decemlineata* (Coleoptera: Chrysomelidae)	Orono, Maine	1978
ARSEF 356	(Orthoptera: Acrididae)	New South Wales, Australia	1979
ARSEF 357	(Orthoptera: Acrididae)	New South Wales, Australia	1979
ARSEF 534	*L. decemlineata* (Coleoptera: Chrysomelidae)	Ithaca, New York	1980
ARSEF 535	*L. decemlineata* (Coleoptera: Chrysomelidae)	Ithaca, New York	1980
ARSEF 792	*Blissus leucopterus* (Hemiptera: Lygaeidae)	Riley, Kansas	1981
ARSEF 1038	*Strinia nubilalis* (Lepidoptera: Pyralidae)	Tully, New York	1983
ARSEF 1039	*L. decemlineata* (Coleoptera: Chrysomelidae)	Tully, New York	1983
ARSEF 1053	(Orthoptera: Acrididae)	Parana, Brazil	1983
ARSEF 1079	*L. decemlineata* (Coleoptera: Chrysomelidae)	Washington	1984
ARSEF 1326	(Orthoptera: Acrididae)	Pyrenees-Orientales, France	1983
ARSEF 1619	*L. decemlineata* (Coleoptera: Chrysomelidae)	Tully, New York	1984
ARSEF 1689	From formulated product (Abbott Laboratories)		1984
ARSEF 1707	*L. decemlineata* (Coleoptera: Chrysomelidae)	Tully, New York	1984
ARSEF 1959	(Orthoptera: Acrididae)	Bahia, Brazil	1985
ARSEF 2054	*Lymantria dispar* (Lepidoptera: Lymantriidae)	Dexter, Oregon	1985
ARSEF 2430	*Blissus leucopterus* (Hemiptera: Lygaeidae)	Wakefield, Kansas	1980
ARSEF 2580	*Coccinella septempunctata* (Coleoptera: Coccinelidae)	Niles, Michigan	1988
ARSEF 2860	*Schizaphis graminum* (Homoptera: Aphididae)	Parma, Idaho	1987
ARSEF 2861	*Diuraphis noxia* (Homoptera: Aphididae)	Parma, Idaho	1988
ARSEF 2976	*O. nubilalis* (Lepidoptera: Pyralidae)	Centre, Pennsylvania	1990
ARSEF 3037	*Anthonomus musculus* (Coleoptera: Curculionidae)	Carver, Massachussetts	1990
ARSEF 3111	*Diabrotica virgifera* (Coleoptera: Chrysomelidae)	Mead, Nebraska	1990
ARSEF 4524	(Homoptera: Aphididae)	Ontario, New York	1994
ARSEF 4534	*Plutella xyllostella* (Lepidoptera: Plutellidae)	Ontario, New York	1993
ARSEF 5533	*L. decemlineata* (Coleoptera: Chrysomelidae)	Freeville, New York	1997
ARSEF 5537	*L. decemlineata* (Coleoptera: Chrysomelidae)	Freeville, New York	1997
ARSEF 6444	GHA reisolated from *Locusta migratoria* (Orthoptera: Acrididae)		2000
NY 1	*L. decemlineata* (Coleoptera: Chrysomelidae)	Freeville, New York	2000
NY 2	*L. decemlineata* (Coleoptera: Chrysomelidae)	Freeville, New York	2000
NY 4	*L. decemlineata* (Coleoptera: Chrysomelidae)	Freeville, New York	2000
NY 5	*L. decemlineata* (Coleoptera: Chrysomelidae)	Freeville, New York	2000
NY 9	*L. decemlineata* (Coleoptera: Chrysomelidae)	Freeville, New York	2000
NY 11	*L. decemlineata* (Coleoptera: Chrysomelidae)	Freeville, New York	2000
NY 15	*L. decemlineata* (Coleoptera: Chrysomelidae)	Freeville, New York	2000
B. amorpha			
ARSEF 2251	Coleoptera	Para, Brazil	1980
ARSEF 2641	*Solenopsis* sp. (Hymenoptera: Formicidae)	Sao Paulo, Brazil	1988
B. brongniartii			
ARSEF 617	*Melolontha melolontha* (Coleoptera: Scarabaeidae)	France	1981
ARSEF 1068	*M. melolontha* (Coleoptera: Scarabaeidae)	Thurgau, Switzerland	1982
Metarhizium anisopliae			
ARSEF 1280	*Popillia japonica* (Coleoptera: Scarabaeidae)	Clinton Corners, NewYork	1978
ARSEF 2547	*Rhizotrogus majalis* (Coleoptera: Scarabeaidae)	Syracuse, New York	1987
ARSEF 2548	*P. japonica* (Coleoptera: Scarabaeidae)	Sleepy Hollow, New York	1987

purified using Qiagen anion-exchange resins, followed by restriction digest with *Eco*R1 to recover the insert DNA. The cloned fragments were sequenced by the BioResource Center at Cornell University, Ithaca, NY using BIG Dye Terminator chemistry and AmpliTaq-FS DNA polymerase (Applied Biosystems). The nucleotide sequence of the cloned RAPD fragments from OPA-5, OPA-14, OPA-15, and OPB-9 have been deposited in the GenBank database under Accession Nos. BH190223, BH190224, BH190225, and BH190226, respectively.

2.4. SCAR primers and PCR conditions

The nucleotide sequence of each of the cloned RAPD fragment was used to design pairs of SCAR primers (Table 2). These primers were synthesized by the BioResource Center at Cornell University, Ithaca, NY. PCR mixtures (50 μl volume) for SCAR assays consisted of 1× Expand High Fidelity buffer (10 mM Tris–HCl, 50 mM KCl at pH 8.3) with 1.5 mM $MgCl_2$ (Roche); 200 μM each of dATP, dCTP, dGTP, and dTTP (Roche); 0.5 μM of each primer (forward and reverse primers); 20 ng fungal DNA; and 2.6 U of Expand High Fidelity PCR System enzyme mix (*Taq* and *Pwo* DNA polymerases) (Roche). The amplification profile was 2 min initial denaturation at 94 °C; 10 cycles of denaturation at 94 °C for 15 s, annealing at 63 °C for 30 s, and elongation at 72 °C for 45 s; followed by 15 cycles of denaturation at 94 °C for 15 s, annealing at 63 °C for 30 sec, and elongation at 72 °C for 45 s, with an additional 5 s for each successive cycle; and a final elongation at 72 °C for 7 min. PCR products were visualized in 1% agarose gels stained with ethidium bromide.

Table 2
Sequence of SCAR primers derived from RAPD markers diagnostic for *B. bassiana* strain GHA[a]

SCAR markers[b]	Primers[c]	Sequence (5′–3′)[d]
$SCA14_{445}$	OPA14 F_{445}	TCT GTG CTG GCC CTT ATC G
	OPA14 R_{445}	TCT GTG CTG GGT ACT GAC GTG
$SCA15_{441}$	OPA15 F_{441}	TTC CGA ACC CGG TTA AGA GAC
	OPA15 R_{441}	TTC CGA ACC CAT CAT CCT GC
$SCB9_{677}$	OPB9 F_{677}	TGG GGG ACT CGC AAA CAG
	OPB9 R_{677}	TGG GGG ACT CAC TCC ACG

[a] SCAR primer pairs derived from RAPD amplicon OPA-$5_{0.49}$ were highly polymorphic and were not included for further analysis.
[b] Subscript refers to the size of the amplified product from *B. bassiana* strain GHA genomic DNA.
[c] Primer nomenclature is according to Paran and Michelmore (1993). Primer name refers to the Operon Technologies primer kit and number, followed by the letters F and R to indicate forward and reverse primer, respectively, and a subscript denoting size of amplification product. Each primer set is based on the sequence of both ends of the cloned RAPD fragment.
[d] Underlined nucleotides indicate sequences of the corresponding RAPD primers.

Specificity of each SCAR primer pair was tested by PCR assays against fungal strains used in the RAPD screening. Isolates of *B. bassiana* collected from mycosed Colorado potato beetles from GHA-treated plots in September 2000 in Freeville, NY, were also included in SCAR assays to identify the *B. bassiana* strain(s) infecting these beetles. Using a sterile loop, conidia from infected beetles were collected, suspended in 600 μl sterile distilled water containing 0.2% Tween 80, and 200-μl aliquots were plated on three oatmeal dodine agar (ODA) plates for isolation of *B. bassiana* colony forming units (CFU) (Chase et al., 1986; Costa and Gaugler, 1989). ODA plates were incubated as described above. Six *B. bassiana*-like CFUs on ODA plates were cultured for DNA extraction and assayed with SCAR primers to detect presence of *B. bassiana* GHA.

All SCAR assays were replicated thrice. SCAR primers pairs derived from OPA-5 amplicon were found to be highly polymorphic, and, consequently, were eliminated for further screening. PCR products generated by SCAR primers derived from OPA-14, OPA-15, and OPB-9 amplicons were recovered from agarose gels using QIAquick gel extraction kit (Qiagen) and were digested with restriction enzymes *Hae*II or *Hin*dIII, *Bsr*F1, and *Bsr*F1 or *Dra*I, respectively, to verify whether DNA fragments obtained from other strains of *B. bassiana* or from isolates from infected beetles in GHA-treated plots were identical to the *B. bassiana* GHA amplicons.

The sensitivity of devised SCAR assays was tested by setting up PCR with variable quantities (1 pg to 100 ng) of *B. bassiana* GHA genomic DNA. A possible inhibitory effect on PCR amplification by contaminant DNA was tested by spiking a separate set of these PCR with 50 ng *B. bassiana* NY1, a strain found to be distinct from GHA in preliminary assays. Sensitivity assays were replicated at least twice.

2.5. Detection of B. bassiana strain GHA in field sample: plating method

Soil and potato foliage samples from experimental plots in Freeville, NY, were collected in late June 2001, prior to and immediately after spraying of GHA-based Mycotrol ES (Emerald BioAgriculture) in the summer of 2001. Samples were collected prior to spraying to establish background levels of indigenous *B. bassiana* strains present, as well as the strain GHA since these plots were used for experiments on field efficacy of GHA formulated products during previous years (Wraight and Ramos, 2002). Using a soil corer (8 cm diameter), three soil samples (approximately 5 cm in depth) were collected along a transect of each of three plots. From each sample, 25 g soil (loam, pH 5.4–5.8) was mixed with 25 ml sterile distilled water with 0.2% Tween 80 for 5 min using a vortexer. Serial dilutions (10^{-1}–10^{-4}) of the soil slurry were prepared, and a 200-μl aliquot was

plated on each of three ODA plates. Fungi from potato foliage were sampled by immersing three leaf disc samples (2 cm diameter) from individual leaves of a given potato plant in 20 ml sterile distilled water containing 0.2% Tween 80 followed by vortexing for 5 min to dislodge conidia. Three leaves were sampled per plant, with three plants sampled from each of three plots. Dilutions were prepared and aliquots plated as described above.

Another extraction method was adopted for isolating genomic DNA for the detection of *B. bassiana* GHA from field samples. A loopful of conidia from individual *B. bassiana*-like CFUs on ODA plates was cultured in 2 ml PD broth (10 g bacto peptone and 20 g dextrose/L) in a 15-ml round-bottomed polypropylene tube with shaking (150 rpm) at room temperature (~24 °C). After 3 days of growth, 2 ml YPD broth (PD broth plus 2 g yeast extract/L) were added, and the culture was incubated for another 24 h. A 1.5-ml aliquot of fungal culture was transferred into a 2-ml microcentrifuge tube for DNA extraction. A small-scale extraction protocol using Wizard Genomic kit (Promega) was adopted following the manufacturer's yeast protocol with the following modification: 150 μg lyticase was replaced with 10 mg each of *A. niger* cellulase and *T. harzianum* cellulase.

2.6. Detection of B. bassiana strain GHA in field sample: direct culture method

An alternative procedure to the plating method for processing environmental samples was tested. Undiluted soil wash was directly inoculated into the liquid medium to detect any *B. bassiana* propagule present. Four milliliters of PD broth supplemented with dodine (Cyprex, 65% AI, 100 mg/L) and antibiotics (70 mg penicillin and 100 mg streptomycin sulfate/L) was inoculated with one milliliter of soil washing from the same soil suspension sampled for CFUs on ODA plates and incubated at room temperature (~24 °C) with shaking (150 rpm). After 7 days, 5 ml YPD broth was added and the fungal culture was incubated for another 24 h. A 1.5-ml aliquot was collected for DNA extraction following the modified Wizard yeast protocol (Promega). In cultures with sparse fungal growth, an additional 1.5-ml aliquot was collected. Five microliters of DNA solution (from total volume of 20 μl) extracted from liquid cultures of either CFUs or soil washings was used in SCAR assays as described in this paper.

3. Results and discussion

3.1. RAPD analyses of B. bassiana GHA and other entomopathogenic fungi

Of the 88 RAPD primers screened, 55 generated PCR products unique to GHA among the nine *B. bassiana* strains used for initial screening. Among the 55 primers, four were selected for generating well-resolved and reproducible bands that were less than 1 kb and thus easily amenable to cloning and sequencing. The selected RAPD primers OPA-5, OPA-14, OPA-15, and OPB-9 produced unique amplicons of 494 (OPA-$5_{0.49}$), 445 (OPA-$14_{0.44}$), 441 (OPA-$15_{0.44}$), and 677 (OPB-$9_{0.67}$) bp in *B. bassiana* GHA (Fig. 1). Further screening against other strains of *B. bassiana* and other entomopathogenic fungi revealed that RAPD primer OPA-14 generated a DNA fragment in ARSEF 350 of similar size to the GHA amplicon (Fig. 1B). With primers OPA-5, OPA-15, or OPB-9 PCR products of similar size to the GHA amplicon were generated by two or more *B. bassiana* strains: for OPA-5 or OPB-9, ARSEF strains 32 and 792 (data not shown); and for OPA-15, ARSEF strains 318, 2430, 2860, and 2861 (Fig. 1C). There was, however, no *B. bassiana* strain among the 43 tested in this study that showed all four diagnostic RAPD markers similar to those in strain GHA other than ARSEF 6444, a reisolate of *B. bassiana* GHA from *Locusta migratoria*. ARSEF 6444 also had a RAPD profile identical to GHA for all the RAPD primers tested (data not shown). All four single spore isolates of GHA from the technical product had identical RAPD profiles (Fig. 1).

Although differentiation of GHA from 41 other strains of *B. bassiana* was readily accomplished using the RAPD markers, this technique required the establishment of single spore isolates, making detection in field samples very laborious and time consuming. Conversion of RAPD amplicons that distinguished GHA into molecular probes specific for this strain would facilitate detection in mixed field samples whether from mycosed insects or environmental samples such as soil and foliage.

3.2. Development of SCAR markers

To develop a diagnostic assay for *B. bassiana* GHA the four selected RAPD markers were converted to SCAR markers. A pair of SCAR primer, forward and reverse primers, was designed based on the sequence of both ends of each of the four cloned RAPD markers. Primer pair sequences, except for those derived from OPA-$5_{0.49}$ amplicon, which were found highly polymorphic, are listed in Table 2. Primer sequence consisted of the 10-nucleotide sequence of the corresponding RAPD primer, followed by a variable number of bases of the amplified sequence. Primer length was determined by compatibility of melting temperatures of the forward and reverse primers.

PCR assays of *B. bassiana* GHA with SCAR primers resulted in DNA fragments of the same size as the cloned RAPD amplicons as expected based on primer design. PCR assays of 42 other strains of *B. bassiana*, two strains of *B. amorpha*, two strains of *B. brongniartii*,

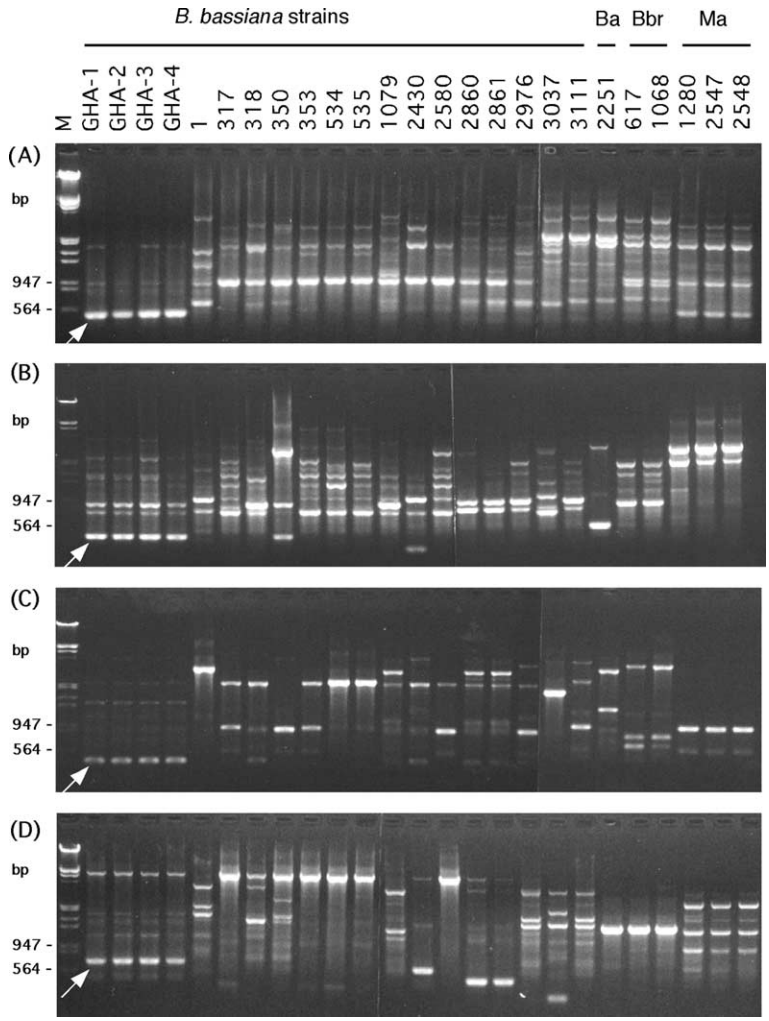

Fig. 1. PCR amplification of strains of *B. bassiana*, *B. amorpha* (Ba), *B. brongniartii* (Bbr), and *Metarhizium anisopliae* (Ma) with RAPD primers (A) OPA-5, (B) OPA-14, (C) OPA-15, and (D) OPB-9. Arrows indicate diagnostic amplicons of 495, 445, 441, and 677 bp for strain GHA with primers OPA-5, OPA-14, OPA-15, and OPB-9, respectively. Fungal strains other than GHA are listed by their ARSEF culture collection number. Lane M, molecular weight markers (*Eco*RI and *Hin*dIII digested bacteria phage Lambda DNA; Life Technologies).

three strains of *M. anisopliae*, and six *B. bassiana* isolates from infected Colorado potato beetle cadavers showed that SCAR primer OPA14 F/R_{445} was specific to *B. bassiana* GHA. A 445-bp DNA fragment was generated only in GHA, in ARSEF 6444, and in isolates collected from field samples from GHA-treated plots (Fig. 2A). Identity of DNA fragments was verified by use of restriction enzymes *Hae*II or *Hin*dIII (data not shown). The restriction enzymes *Hae*II and *Hin*dIII each recognize one site in the 445 bp DNA sequence, sites 60 and 136 bp, respectively, generating two fragments upon cleavage. All GHA-based isolates were observed to generate these DNA fragments (data not shown).

SCAR primers OPA15 F/R_{441} and OPB9 F/R_{677}, however, were polymorphic, generating bands of similar size to the GHA amplicons in other *B. bassiana* strains (Figs. 2B and C) even after several attempts to optimize PCR conditions and increase specificity. Bands generated in other *B. bassiana* strains were verified to be non-specific by use of restriction enzyme *Bsr*F1 and *Bsr*F1, or *Dra*I for the 441 and the 677 bp amplicons generated by OPA15 F/R_{441} and OPB9 F/R_{677}, respectively. DNA fragments in other *B. bassiana* strains used in this study showed different restriction patterns compared to strain GHA (data not shown).

It is noteworthy that two of six *B. bassiana* isolates, A1-a and A1-b, recovered from mycosed beetles from experimental plots sprayed with GHA in Freeville, NY, in September 2000, and included in SCAR assays, were not GHA (Fig. 2A). RAPD analysis of single spore isolates from A1-a and A1-b revealed that they were identical to the most common indigenous strain in Freeville, NY, represented by NY1 (L. Castrillo, unpublished data). In a separate RAPD analysis of isolates collected in July 1997 and in July 2000, data revealed the presence of this common genotype among strains associated with Colorado potato beetles in Freeville, NY (L. Castrillo, unpublished data). The presence of indigenous

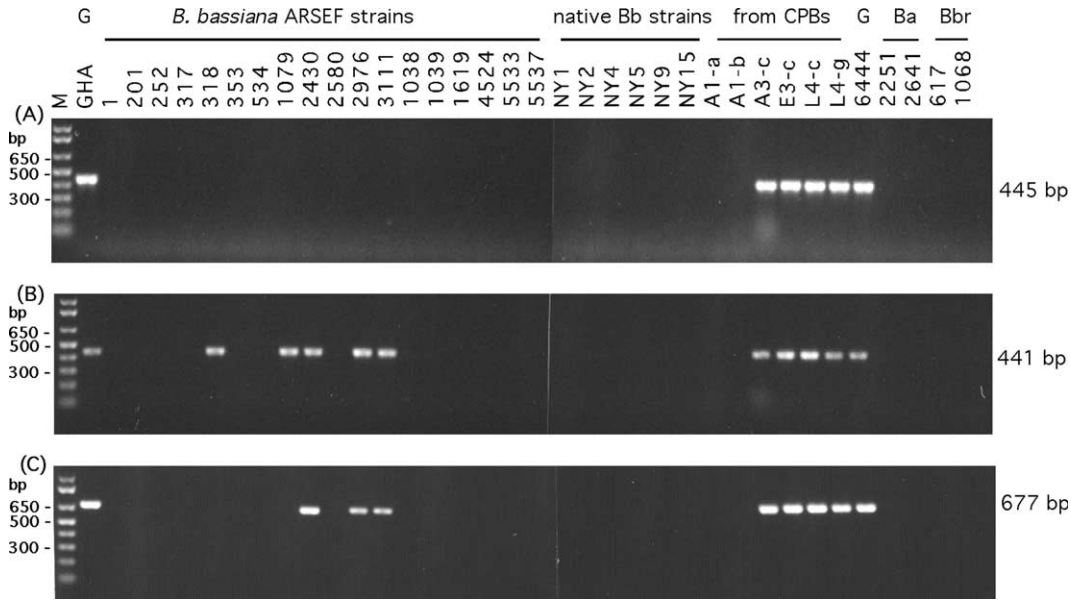

Fig. 2. PCR assays of *B. bassiana* strain GHA (G) and other strains of *B. bassiana* (Bb), *B. amorpha* (Ba), and *B. brongniartii* (Bbr) with SCAR primers (A) OPA14 F/R_{445}, (B) OPA15 F/R_{441}, and (C) OPB9 F/R_{677}. Primer OPA14 F/R_{445} was specific to *B. bassiana* GHA, generating the 445 bp amplicon only in GHA, ARSEF 6444 (a reisolate of GHA), and isolates from mycosed Colorado potato beetle (CPB) cadavers collected from GHA-treated plots (isolates A3-c, E3-c, L4-c, and L4-g). No PCR product was generated in any of the non-GHA based isolates by primer OPA14 F/R_{445}. Primers OPA15 F/R_{441} and OPB9 F/R_{677} were polymorphic, generating the 441 and the 677 bp amplicon, respectively, in GHA and GHA-based isolates as well as in a few other strains of *B. bassiana*. Lane M, molecular weight markers (1 kb Plus DNA ladder; Life Technologies).

strains of *B. bassiana* among those collected from treated plots underscores the importance of identifying *B. bassiana* isolates collected after field application to corroborate efficacy studies of an introduced strain.

The presence of indigenous strains also raised the question of whether the limit of detection of *B. bassiana* GHA propagules in mixed field samples by use of devised SCAR assays will be affected by non-target or contaminant DNA. Non-target DNA may cause reduced sensitivity of detection resulting in false-negative reactions (Wilson, 1997). Sensitivity assays with variable quantities of GHA genomic DNA showed that as little as 100 pg GHA genomic DNA was sufficient to generate easily visible bands with each of the three SCAR primers whether GHA genomic DNA was pure or contaminated with strain NY1 (data not shown).

The sensitivity of devised SCAR assays for *B. bassiana* strain GHA even in the presence of contaminant DNA indicated that these assays could be used to detect GHA in mixed cultures from environmental samples. However, the possibility of non-specific bands generated by SCAR primers OPA15 F/R_{441} and OPB9 F/R_{677} limits their use to cases where it has been shown that these primers do not anneal to any of the indigenous *B. bassiana* strains. With primer OPA14 F/R_{445}, the specificity and sensitivity of devised SCAR assay permit diagnostic detection of variable levels of GHA propagules in mixed field samples. The exact limit of detection of propagules in terms on conidia per gram of soil, however, will be difficult to estimate because field samples obtained for SCAR detection need to be cultured on selective medium prior to obtaining fungal DNA for analysis.

3.3. Detection of B. bassiana GHA in field samples

Beauveria bassiana propagules were recovered from soil and foliage samples collected from potato plots after application of a GHA-based mycoinsecticide, but not from samples collected prior to spraying in the summer of 2001. The absence of indigenous strains of *B. bassiana* may be attributed to the limited number of samples collected and the very low number of Colorado potato beetles observed during this sampling period. Previous samples of indigenous strains of *B. bassiana* were collected from mycosed beetles. The absence of GHA from these plots, with a history of GHA application, may be due to factors relating to field persistence of GHA and also to the limited number of samples collected.

The number of *B. bassiana* CFUs observed on ODA plates ranged from 5.0×10^2 to 2.4×10^3 CFUs/g soil sample and 7.1×10 to 2.6×10^4 CFUs/cm^2 leaf sample. All representative CFUs assayed with SCAR marker OPA14 F/R_{445} revealed the presence of *B. bassiana* GHA. The use of SCAR markers facilitated the detection of GHA in CFUs from environmental samples without the need for establishment of single spore isolates. SCAR assays of CFUs also provided additional information on field collected isolates compared to dilution plating alone, which provided propagule

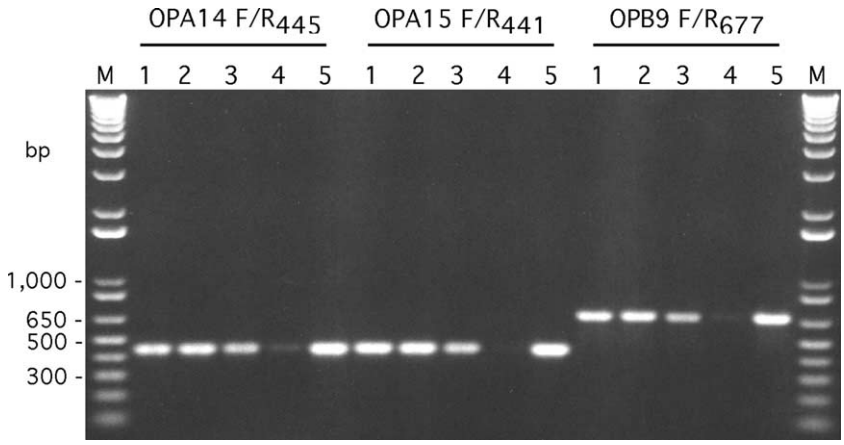

Fig. 3. PCR amplification with SCAR primers OPA14 F/R_{445}, OPA15 F/R_{441}, and OPB9 F/R_{677} of genomic DNA extracted from fungal cultures from soil suspensions inoculated into a semi-selective liquid medium for *B. bassiana*. Shown are PCR products from liquid cultures from four soil samples with variable amounts of *B. bassiana* GHA propagules (lanes 1–3 with 10^3 CFUs/g soil; lane 4 with 10^2 CFUs/g soil) and from a liquid culture inoculated with Mycotrol ES containing $\sim 10^6$ conidia of *B. bassiana* GHA (lane 5). Lane M, molecular weight markers (1 kb Plus DNA ladder; Life Technologies).

estimates only. Thus, a combination of SCAR assays and dilution plating can be used for studies verifying identity of the etiological agent and studies tracking the level of inoculum persisting in the environment after application. Although CFUs cannot be directly equated with number of spores present because a given CFU can be based on more than a single conidium or mycelial fragments, the number of CFUs sampled over time provide an estimate of and show trends in fungal population numbers.

Direct culture of soil washings in liquid medium provided an alternative method of detecting the presence of GHA in soil samples. SCAR assays of genomic DNA from fungal cultures from soil washings all showed DNA fragments corresponding to the specific product for each SCAR primer (Fig. 3). Intensity of bands varied and correlated with the results of dilution plating method. Soil inoculum that revealed 10^3 CFUs/g soil generated more intense bands than soil samples with 10^2 CFUs. Direct correlation of band intensity with counts of CFUs cannot be used, however, because SCAR assays utilize a small sample of the genomic extract and inherent variability in the sequence of steps entailed to obtain these samples can confound sampling errors. Thus, quantification of a PCR product cannot be directly equated with the number of fungal propagules present in the soil sample (Wilson, 1997). Nonetheless, SCAR assays of cultures from soil washings allow for simple testing of environmental samples for absence or presence of GHA and can be used for studies involving large number of samples.

Both methods described for detection of GHA in field samples still required culture in selective media prior to extraction of genomic DNA. The development of DNA extraction methods for fungi in soil samples (Kuske et al., 1998; Volossiouk et al., 1995) would enhance and hasten detection by obviating the need for culture in artificial media. However, these methods do not ensure viability of detected propagules, making these methods of little value in persistence studies because the presence of a propagule cannot be assumed to count as a source of inoculum for infection.

In summary, the conversion of RAPD markers that differentiated *B. bassiana* GHA from other strains of the fungus into GHA-specific SCAR markers facilitated the detection of GHA propagules from environmental samples. SCAR assays obviated the need for establishment of single spore isolates and the combination of SCAR assays with dilution plating method allowed for the detection and estimation of GHA propagules in field samples for efficacy and persistence studies.

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