DEVELOPMENT OF *BEAUVERIA BASSIANA* FORMULATIONS AND GENETICALLY MARKED STRAINS AS A POTENTIAL BIOPESTICIDE FOR IMPORTED FIRE ANT CONTROL

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ABSTRACT

A strain of *Beauveria bassiana* formulated as alginate pellets and broadcast in field plots significantly reduced ratings of *Solenopsis invicta* colonies as compared to control treatments during two seasons of trials. A coating of peanut oil on mycelial pellets greatly enhanced retrieval of pellets by *S. invicta* foragers. *Solenopsis invicta* individuals collected in fungal-treated plots and held in the laboratory died of *B. bassiana* mycoses; whereas, no *B. bassiana* disease was detected in ants from control plots. Genetically tagged strains of *B. bassiana* carrying an antibiotic resistance gene and the β-glucuronidase gene product will serve to identify the fungus when it is used for biocontrol against *S. invicta*. Tests on the insecticidal capability of the reporter strains in the laboratory indicate that they are as effective as the wild-type parental strain and can, therefore, be used in field studies.

INTRODUCTION

Beauveria bassiana (Bals.) Vuill. (Fungi: Deuteromycotina) causes mortality of imported fire ants in the laboratory and under field conditions. Broome (1974) applied *B. bassiana* to *Solenopsis richteri* Forel (Hymenoptera: Formicidae), the black imported fire ant, and achieved 90% mortality of larvae and 67% mortality of adults when applied orally and 35% and 72% when applied to cuticle of larvae and adults, respectively.

Quattlebaum (1980) successfully infected *Solenopsis invicta* Buren (Hymenoptera: Formicidae), the red imported fire ant, colonies in South Carolina by feeding *B. bassiana*-infected *Heliothis* sp. (Lepidoptera: Noctuidae) larval cadavers. Mortality of treated colonies ranged between 21.7 and 47.1% when compared to control colonies where mortality ranged between 4.3 and 12.3%.

Stimac et al. (1993) reported 70 to 92% mortality of *S. invicta* after injection of a powder formulation of *B. bassiana* conidia and diatomaceous earth into mound soil. Also, a concentration of 10^6 conidia per gram of sterile soil caused 90% *S. invicta* mortality; whereas, no significant ant mortality was measured in non-sterile soil.

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Oi et al. (1994) applied *B. bassiana* to *S. invicta* mounds in Florida pastures in one of three ways: a culture of *B. bassiana* grown on cooked rice and applied to the tops of mounds; injections of conidial powder formulations; and injections of *B. bassiana* conidia mixed with diatomaceous earth. Rice inoculaltions resulted in maximum infection rate of 55% of live *S. invicta* sampled.

Solenopsis invicta adults were exposed to *B. bassiana* conidia in directed spray, total water immersion, direct contact to contaminated surfaces, in inoculated sand, and mixed in baits (Siebeneicher et al. 1992). All application techniques caused significantly greater mortality than those in control treatments. Germinated conidia were found on adult tarsi after 120 h, in crops of larger workers, and in buccal pellets. Consequently, tarsi and the buccal cavity of the head were considered the major routes of infection. Fourth-stage larvae were fed contaminated buccal pellets by workers and became infected. However, extensive grooming, removal of dead ants from colonies, and antifungal activity of venom may have limited epizootic development in *S. invicta* colonies and populations (Siebeneicher et al. 1992).

A *B. bassiana* strain was isolated from *Atta mexicana*, the Mexican leaf-cutting ant, and was reisolated several times from infected *S. invicta* (Sanchez-Pena 1992). In the laboratory, this *B. bassiana* strain caused *S. invicta* mortality when applied as conidia and as dried mycelia. Dried mycelia were added to commercial potting soil and tested against *S. invicta* queens. As little as 0.005% of *B. bassiana* added to soil (w:w) caused 63.3% mortality after 19 days.

Encapsulation of biological control agents in an alginate matrix may allow greater survivability and viability under field conditions. Alginate is an easily gelled, polysaccharide gum extracted from seaweed and is widely used in food products, pharmaceuticals, cosmetics, and industry (McNeely and Pettitt 1973).

The first reported use of sodium alginate to encapsulate a living biological control agent was to create a mycoherbicide by encapsulating five different fungi (Walker and Connick 1983). An isolate of *B. bassiana* was alginate-encapsulated and caused considerable cereal aphid mortality under laboratory conditions (Knudsen et al. 1990).

In small containers of potting soil, *B. bassiana* mycelia in alginate pellets caused 50% mortality of *S. invicta* after 10 days and 90% after 13 days, both significantly greater mortality than those of control treatments (White 1995). Bextine (1998) broadcast *B. bassiana* alginate pellets over pastures heavily infested with *S. invicta* in northeastern Texas. A thin coating of peanut oil on pellets improved retrieval and removal of pellets by *S. invicta* foragers. Results of portions of this study are reported herein.

Field trials of various alginate-encapsulated *B. bassiana* formulations of our strain are being planned with independent cooperating scientists to validate our very promising results. Another goal is to genetically mark our selected *B. bassiana* genome to be able to identify our fungus in dead insect specimens collected in the field. This procedure will ensure accountability that the biological control organism that we introduce into an ecological community targets *S. invicta* populations.

MATERIALS AND METHODS

Fungal Growth and Formulations. Beauveria bassiana was grown on either YP (2.0% yeast extract, 1.0% peptone) or Sabouraud dextrose broth plus 1.0% yeast extract (SDBY) at 30°C. When appropriate, the antibiotic BASTA was used at a final concentration of 700.0 g/ml. Formulations were prepared by separating mycelia from SDBY and mixing mycelia with 1.0% sodium alginate at the rate of 37.0 g of wet mycelia per 100.0 ml of sodium alginate. The suspension was mixed in a blender with 2.0 g wheat bran, then added dropwise to 0.25 M aqueous calcium gluconate. The resulting

pellets were removed after 5 min with a sieve and were dried on double-thickness waxed paper at room temperature. When dried, pellets were about 10% of the volume of wet pellets. Each batch of pellets was tested for fungal viability before field trials by placing a representative sample upon water-moistened filter paper in petri dishes and observing mycelial hydration and conidiogenesis. Oil-coated pellets were produced by rolling 2.0 g of pellets in 2.0 g of commercial peanut oil just before field application. For 1998 trials, a treatment was broadcast using pellets that had been produced by substituting sterile, shredded, filter-paper fiber instead of mycelia and was considered a pellet control treatment.

Field Trials. In a 1997 field trial in Morris Co., Texas, a 3x3 Latin square was established in a grazed pasture with abundant *S. invicta* mounds. Each treatment grid was approximately 700 m^2. Prior to treatment (20 June), each mound within plots was activity rated between 1 and 25 (Lofgren and Williams 1982) and marked with a flag. Three treatments were made: peanut oil-coated, *B. bassiana* pellets; *B. bassiana* pellets without oil coating; no pellet application (control). Pellets were mixed with playground sand and distributed using a hand-held broadcast spreader. Application rates for pellets were 1120.0 g per ha. Control treatments received only sand. All mounds within plots were activity rated four times within the next 13 weeks. Foraging *S. invicta* were collected at each evaluation date in bait cups and were held in the laboratory for observation of mycoses.

In 1998, three replications of 3x3 Latin squares were designed on a 45.7 x 45.7-m grid. The three treatments were: peanut oil-coated *B. bassiana* pellets; peanut oil-coated paper pellets; and no pellets (control). Pellets were spread evenly in plots using a hand-held broadcast spreader at the rate of 1120.0 g per ha. Mound activity ratings were taken just prior to treatments (20 March) and were recorded every two weeks for 12 weeks. Foraging *S. invicta* were collected at each evaluation date in bait cups and were held in the laboratory for observation of mycoses.

Mean activity ratings were calculated for each experiment, and comparisons among treatments were made with analysis of variance (ANOVA) with the critical *P*-value set at 0.05. Mounds with no ant activity were assigned a rating of zero.

Genetic Manipulations. The fungal transformation expression vector, pBARGPE1 (Fig. 1) was kindly provided by Dr. Krishna Podila, Michigan Technological University. The vector has the *Aspergillus nidulans gpd*A promoter and the *A. nidulans trp*C terminator flanking a multiple cloning site. The ß-glucuronidase gene (GUS) was kindly provided by Dr. Randy Allen, Texas Tech University. The 1.8 kb GUS gene was excised by restriction enzyme digestion of pRTL2-GUS as a *BamH*1-*EcoR*1 fragment. The *BamH*1 site was blunt-ended using T4 DNA polymerase. Transformation vector pBARGPE1 was digested with *Xho*1, blunt-ended, and digested with *EcoR*1. The GUS gene was thus directionally cloned under control of the *gpd*A promoter using T4 DNA ligase. The resulting 7.3 kb plasmid construct was transformed into *Escherichia coli* DH5α using $CaCl_2$ transformation (Sambrook et al. 1989).

Spheroplast Production. Spheroplasts of *B. bassiana* were generated using a procedure described by Pfiefer and Khachatourians (1992). Briefly, *B. bassiana* was grown on Sabourand dextrose agar (SDA) for 8-10 d at 30°C. Conidia were harvested from the plates using sterile water and adjusted to a density of approximately 10^8 conidia per ml. Sabourand dextrose broth, (SDB; 200 ml) was inoculated at 1% v/v with the conidial suspension. Cultures were shaken at 30°C for seven days or until blastospores were observed using microscopy. The culture was poured through sterile cheesecloth to separate out the mycelium. Blastospores were harvested by centrifugation using a Sorvall

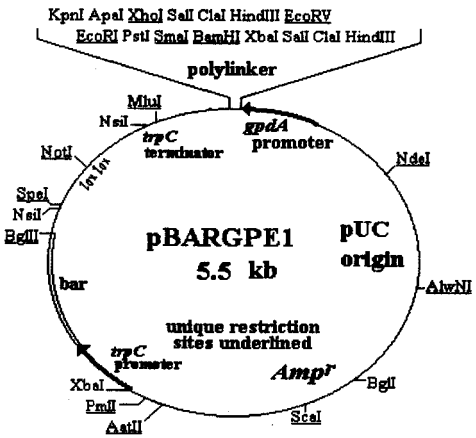


FIG. 1. Schematic of the plasmid vector into which the β-glucuronidase gene was cloned before transformation into *B. bassiana*.

GSA rotor in a RC-5B high-speed centrifuge at 10 k rpm for 30 min at 4°C. The supernatant was carefully decanted, and the blastospores were washed with WASH buffer (0.85% NaCl, 2 mM KH_2PO_4, 4 mM Na_2HPO_4) and re-centrifuged. The blastospores were adjusted to approximately 10^8 blastospores per ml (determined by microscopy). Two hundred ml SDB were inoculated with 0.5 ml of the blastospore suspension that was incubated and shaken overnight at 30°C. The germinating blastospores were washed twice with WASH buffer, weighed, and re-suspended at 5.0 mg/ml in 20 mM Tris-HCl (pH 7.0), 10 mM dithiothreitol (DTT) at 30°C for 1 h with gentle shaking. Following the thiol incubation, the suspension was washed with WASH buffer and then re-suspended at 20 mg/ml in ASP buffer (20 mM KH_2PO4, 600 mM KCl (pH 5.8). Cell wall-degrading enzymes [chitinase (0.5% w/v), cellulase (1% w/v), lysozyme (1% w/v), and β-glucuronidase (0.5% v/v)] were added to generate osmotically stable spheroplasts that were incubated unshaken for 3 h at 30°C. Spheroplasts were then washed with 0.6 M sorbitol twice at 5 k rpm for 10 min at 4°C. The spheroplasts were finally re-suspended to 10^8 cells/ml in 0.6M sorbitol and stored on ice until use.

Electroporation of Spheroplasts. The electroporation protocol was a modification of that described by Pfeifer and Khachatourians (1992). Five μg of linear plasmid DNA, pBARGPE1-GUS, was added to a 200-μl aliquot of spheroplasts incubated on ice for 15 min and transferred to a pre-cooled cuvette. Electroporation was carried out using a BTX Electro Cell Manipulator 600, set at 25 μF and 600 ohms. The use of 10-12.5 kV/cm with 0.2-cm cuvettes or 12.5-25 kV/cm with 0.1-cm cuvettes gave the highest transformation efficiencies in *B. bassiana*. After the voltage was delivered, the cuvette was placed on ice for 15 min, and then diluted with 1.0 ml of SDB with 600 mM NH_4SO_4 added as a stabilizer and incubated for 2 h at 30°C. Following incubation, the aliquots were added to 5 ml of soft agar (YP, 0.5% agar, and 600 mM stabilizer) and poured onto pre-poured YP agar plates with 600 mM stabilizer. After 24 h of growth, the plates are

over-laid with YP agar containing 700 μg/ml of BASTA for selection and incubated at 30°C until transformants appeared.

β-glucuronidase Assay. Transformants were sub-cultured onto YP-BASTA plates. Once these cultures were established, 1 cm^2 mycelial mats were excised and used in the GUS assay. The mycelial mats were ground in micro-centrifuge tubes in GUS assay buffer (50 mM $NaPO_4$ [pH 7.0], 10 mM β-mercaptoethanol, 10 mM EDTA, 0.1% Triton X-100) and incubated at 37°C for 20 min. Following this, 5 μl of 0.5 mM potassium ferricyanide, 5 μl of 0.5 mM ferrocyanide, and 1 mM 5-Bromo-4-chloro-3-indolyl-β-D-Glucuronic acid (X-GlcA) were added and incubated overnight at 37°C.

Fluorometric Assay. Mycelia from transformants were ground in lysis buffer (50 mM $NaPO_4$, [pH 7.0], 10 mM β-mercaptoethanol, 0.1% Triton X-100, 0.1% sodium lauryl sarcosine, and 10 mM EDTA), and a Bradford assay was performed to determine protein concentrations of the material used. Ten μl of protein extract was added to 950 μl of lysis buffer along with 1.0 mM 4-methyl-umbelliferyl-β-D-Glucuronide (MUG), and incubated at 37°C. Fifty-μl samples were removed at zero time and ten min, and reactions were terminated with the addition of 950 μl of 0.2 mM Na_2CO_3. Addition of Na_2CO_3 terminated the reaction and developed the fluorescence of methyl umbelliferone, which is about 7-fold as intense at alkaline pH. A Turner Fluorometer Model 112 was used to measure fluorescence and was standardized using a range of 0 to 100 nM concentrations of the methyl umbelliferone derivative.

Fungal Genomic DNA Preparation. Mycelia was scraped from a week-old plate using a sterile spatula and placed in a tissue homogenizer. Five ml of extraction buffer (100 mM Tris [pH 8.0], 10 mM EDTA, 2% SDS, and 100 μg/ml of Proteinase K, 1% β-mercaptoethanol) was added, and the tissue was ground into slurry and incubated at 60°C for 1 h. The salt concentration was then adjusted to 1.4 M with 5 M NaCl, 1/10 volume of 10% cetyl triethyl ammonium bromide (CTAB) was added, and samples were incubated a further 10 min at 65°C. Following the addition of one volume of chloroform: isoamyl alcohol, the solution was gently emulsified by inversion, incubated at 0°C for 30 min, and centrifuged for 10 min at 11 k rpm at 4°C. The top phase was transferred to a clean tube, and 5 M NH_4OAC was added to a final concentration of 3 M, incubated on ice for 1 h, and spun again. The supernatant was transferred to a new tube and incubated with ribonuclease at 0.02 μg/ml for 5 min at room temperature. Then, 0.55 volumes of isopropanol was added to precipitate the DNA, spun immediately for 10 min at 11 k rpm at 4°C. The supernatant was carefully removed, and the DNA pellet was washed twice with 70% ethanol, air dried for 20 min, and re-suspended in 100 μl of 10 mM Tris (pH 8.0), 1 mM EDTA buffer.

Polymerase Chain Reaction (PCR). Primers were designed from the *uid*A gene sequence (encoding β-glucuronidase), to yield a 350-bp fragment. The forward primer was 5'-TTACGTCCTGTAGAAACCC-3', and the reverse primer was 5'-GACATCGGCTTCAAATGG-3'. The PCR was carried out in 100 μl volume reactions, Fisher Buffer B was used along with a final concentration of 1.5 mg/ml $MgCl_2$. Deoxyribonucleotide triphosphates (dNTPs) were added at a final concentration of 200 μM, and 100 pmole of primers were used. Varying concentrations of genomic template were used, 100 ng to 1μg, and 2 units of Fisher Taq polymerase was used. The reaction conditions were as follows: hot start, 5 min at 94°C; denaturing, 1 min at 94°C; annealing, 1 min at 40°C; extension, 1 min 72°C.

DNA Dot Blot. For genomic southern hybridizations, a genomic dot blot was used with a BIORAD Bio-Dot apparatus. Twenty-five μg of genomic DNA and 100 ng of positive control plasmid pBARGPE1-GUS (final volume of 0.5 ml) containing 0.4 M NaOH and 10 mM EDTA were heated for 10 min at 100°C. The DNA samples were

added to the wells after denaturation without vacuum. The vacuum was run until the wells were dry, and then wells were washed with 0.5 ml of 0.5 Tris-HCl and 1.5 M NaCl and run until the wells were dry. The membrane was baked at 80°C for 2 h under a vacuum to immobilize DNA to the solid support.

Hybridization of Dot Blot. Hybridizations were carried out in a Robbins Scientific Model 2000 micro-hybridization incubator. Prehybridization was carried out at 68°C for 2 h using the Sigma Perfect HybPlus Hybridization Buffer. The pBARGPE1-GUS vector was digested with *EcoR*V to yield a 250-base pair fragment to be used as a probe.

A Roche Diagnostics-Random Primed DNA labeling kit was used for the labeling reaction. Twenty-five ng of DNA of the 250 bp probe was denatured by boiling for 10 min and flash cooled on ice for 5 min. Nucleotides dATP, dGTP, and dTTP were added to a final concentration of 25 μM. To this, 2μl of the 10X hexanucleotide reaction mixture was added, with 5 μl of 50 mCi/ml α ^{32}P-dCTP, and 2 units of Klenow fragment of DNA polymerase. The reaction was incubated for 30 min at 37°C, then boiled for 10 min, and flash-cooled for 5 min. The labeling mix was added to the prehybridization solution, and hybridization carried out at 68°C overnight. The blot was washed with 0.5X SSC and 0.1% SDS for 10 min at 68°C three times. The blot was placed on film and exposed at –80°C overnight.

Bioassays. Two percent agar was poured into 200-ml beakers to give a stable medium for *S. invicta.* Water in a micro-centrifuge tube and food in the form of sterile cat food or apple was provided to each treatment and control. One cm^2 mycelial mats from transformants or the wild type fungus were placed in the beakers, and ant mortality was monitored over a period of three weeks. A negative control with no fungus was included in these assays. Assays were conducted at laboratory temperature and humidity. To confirm that death was caused by the *B. bassiana*, the fungus was cultured from dead ants suspended above sterile media.

RESULTS AND DISCUSSION

Field Trials. No differences in mean mound ratings were detected in pre-treatment analysis (Table 1). After 19 d ($F = 2.71$; df = 6, 74; $P = 0.0198$) and continuing to the end of the trial, mean ratings of mounds in plots treated with peanut oil-coated pellets were significantly lower than those in the other treatments. Peanut oil was a good attractant to *S. invicta*, and foragers carried *B. bassiana* pellets into mounds where the fungus produced conidia and caused death of ants. Apparently pellets with no oil covering were not gathered by foragers or were not retained within colony mounds until conidiogenesis.

Solenopsis invicta were collected in bait cups placed at the edges of mounds on d 19 and 30 and were held in the laboratory. One hundred percent of the ants collected from plots treated with peanut oil-coated pellets produced fungi that were identified as *B. bassiana* after conidiogenesis. No ants collected from the other treated plots developed mycosis. These findings suggest that declines in mound ratings could be attributed to disease caused by applied *B. bassiana.*

Mounds in plots treated in 1998 with *B. bassiana* pellets coated with peanut oil were quickly gathered by foraging *S. invicta.* Mound activity ratings in fungal-treated plots (Table 2) were significantly less at 14 d ($F = 28.91$; df = 6, 166; $P = 0.0001$) and at each subsequent date ($P = 0.0001$). At 42 d and 56 d, the mean activity ratings (< 5.0) indicated that no brood was detected; whereas, mounds in other treatment plots were large and had abundant brood (22.2 and 22.3 in paper pellet and control plots, respectively).

Beauveria bassiana mycelia were identified from 30 captured *S. invicta* (55.7 and 26.7% of ants collected on day 14 and 28, respectively). No ant mortality attributed to *B.*

bassiana was detected in other treatments. These evidences suggest that *B. bassiana* was directly responsible for mound decline in this experiment.

TABLE 1. Mean Ratings of *Solenopsis invicta* Mounds after Broadcast Application of *Beauveria bassiana* Alginate Pellets, 20 June – 19 September 1997. RM Farms, Morris Co., Texas.

	Mean mound ratings [a] Days post-treatment [b]				
Treatment [c]	0	19	30	52	91
Peanut oil	21.2a	14.0a	9.0a	7.2a	5.0a
No oil	21.1a	19.6b	20.5b	20.7b	15.7b
Control	20.0a	20.3b	21.7b	18.4b	18.4b

[a] Lofgren and Williams, 1982.

[b] Means followed by the same letter within a column are not significantly different (ANOVA, LSD; df = 6,74; P>0.05)

[c] Alginate pellets with *B. bassiana* coated with peanut oil, alginate *B. bassiana* pellets without peanut oil, or no pellets applied.

TABLE 2. Mean Ratings of *Solenopsis invicta* Mounds after Broadcast Application of *Beauveria bassiana* Pellets on 20 March 1998. Bextine Farms, Morris Co., Texas.

	Mean mound ratings [a] Days post-treatment [b]				
Treatment [c]	0	14	28	42	56
Fungal pellets	20.7a	6.2a	5.1a	3.0a	2.1a
Paper pellets	21.4a	21.4b	21.6b	22.1b	22.2b
Control	21.2a	20.6b	20.6b	21.5b	22.3b

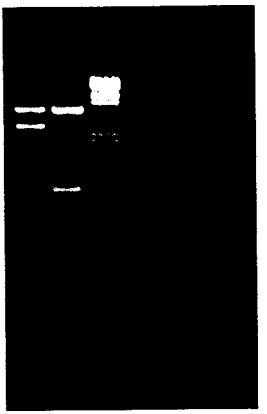
[a] Lofgren and Williams, 1982.

[b] Means followed by the same letter within a column are not significantly different (ANOVA, LSD; df = 6, 165-214; P>0.05)

[c] Alginate pellets with *B. bassiana* coated with peanut oil, alginate pellets with paper fiber coated with peanut oil, no pellets applied.

Generation of a Genetically Tagged Strain of B. bassiana. The use of β-glucuronidase as a reporter gene has been substantially documented in a variety of eukaryotic systems (Jefferson et al. 1987, Couteaudier et al. 1993). The β-glucuronidase gene from *E. coli* was successfully cloned into the fungal vector pBARGPE1 to generate the plasmid pBARGPE1-GUS using restriction enzymes *Not*I and *Eco*R1. Fig. 2 shows the confirming restriction digestion pattern of the vector carrying the β-glucuronidase gene compared to the vector without an insert. Note that the lower band in lane 2 is at a higher molecular weight in lane 1. This indicates the presence of the Gus gene into the smaller fragment yielding a larger one. This construct was used to transform *B. bassiana.*

Transformation of B. bassiana. Spheroplasts were derived from germinating mycelia as described, and 17 different experimental conditions were utilized to optimize electroporation conditions. Out of the seventeen, four treatments resulted in no fungal growth after one week of incubation. This may be due to the high voltages, which killed a majority of the spheroplasts.



1 2 3

FIG. 2. Lane 1, *Not*I and *EcoR*1 digest of pBARGPE1-GUS. lane 2, *Not*I and *EcoR*I digest of pBARGPE1. Lane 3 lambda-*Hind*III digest. Lane 1 shows the increased size of the smaller fragment relative to lane 2. This indicates that the GUS gene has been cloned into that fragment.

To determine which of the remaining isolates contained the sub-cloned GUS gene, a crude GUS assay was performed. Mycelia were ground in GUS assay buffer and incubated with X-GlcA at 37^{0}C overnight. A positive control with a protein extract from *E. coli* was also included. Only three isolates demonstrated strong GUS activity; whereas, two showed marginal activity. After serially sub-culturing the five isolates, only isolates 7A, 7B, 13A, and 13B retained the ability to grow on media containing the anti-fungal antibiotic BASTA. The GUS transformants, the wild type strain, and *E.coli* were all grown under appropriate conditions, and protein extracts were isolated in Lysis buffer as previously mentioned. Ten µl of protein extract quantified by Bradford assay were incubated in MUG assay buffer, and fluorescence was measured (Table 3). Transformant 13A showed twice as much GUS activity as compared to the endogenous background activity of the wild type strain, and transformants 7A, 7B, and 13B showed activity marginally higher than the control un-transformed strain. The GUS gene product, β-glucuronidase, is capable of degrading *B. bassiana* cell walls. Indeed, the enzyme is part of the cocktail of enzymes used in the preparation of spheroplasts (see Materials and Methods). This cell wall-degrading activity may result in reducing the integrity of the fungal cell walls and, therefore, is not expressed at maximal levels in the transformants as observed in the enzyme assays.

Genomic DNA of strain 13A and wild type strain was purified for PCR and Southern hybridizations to definitively show the presence of the GUS gene in the fungal genome. Fig. 3 shows a genomic dot blot of *B. bassiana* transformants compared with wild-type *B. bassiana* and *E. coli* (as a positive control). Positive hybridization with the GUS gene probe, as indicated by autoradiography, was observed with the *B. bassiana* transformants (top row spots 2-5) and the positive control (bottom row spot 1), but not

TABLE 3. GUS Activity of *B. bassiana* Wild Type and Transformed Strains by Monitoring the Production of Methylumbelliferone. Specific Activity Values are Expressed as nmoles of Product Formed per µg protein.

Strain	Time zero	10min.	Specific Activity
7A	27.6	23.0	290
7B	19.1	24.4	290
13A	19.3	38.3	560
13B	17.7	18.1	240
Wild Type	19.2	17.2	200

with the wild type untransformed strain (middle row spots 1 and 2). The relatively strong signal with the positive control compared with the transformed *B. bassiana* is probably due to the high copy number of the plasmid carrying the GUS gene in *E. coli*. In the transformants, the GUS gene probably exists only as a single or few copies, and this could explain the less abundant signal.

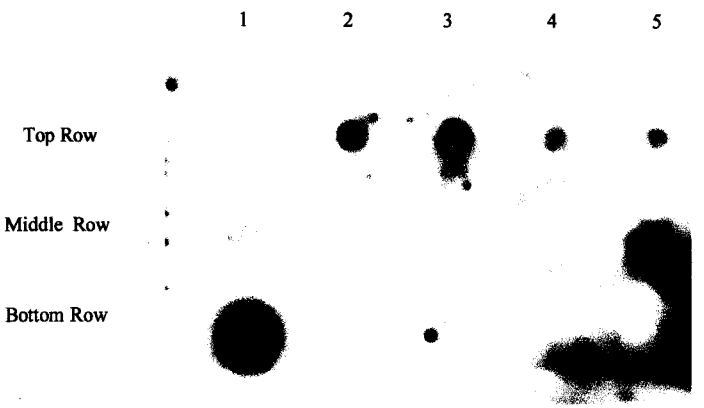


FIG. 3. Genomic dot blot of five isolates of 13A are in the top row, two replicates of wild type *B. bassiana* in the middle row and positive control plasmid DNA pBARGPE1-GUS in the lower left corner of the bottom row. The radiolabeled GUS gene was used as the probe. Positive signals are observed in spots 2-5 of the top row and in the positive control in the bottom row.

Initial studies using the *B. bassiana* GUS transformants indicate that the transformants are effective in killing fire ants in the laboratory environment. Over the assay period of one week, however, we observed that the parental strain of the fungus was more efficient at killing the ants, the mortality rate being slightly higher than with the transformed isolates. The wild type fungus killed 85% percent of the ants in the first three days of the assay. Two of the transformants tested were observed to kill 75 and 60% of the ants within four days, respectively. Over 95% of the ants were killed within one week. In the control with no fungus only 15% of the ants were killed over the first

four days and only 25% were dead after one week. The differential capabilities of the transformants to kill fire ants are expected to vary depending upon the site at which the GUS gene is inserted into the genome of the fungus. Thus it is vital that laboratory assays be carried out before field testing commences. We are now in a position to evaluate the efficacy of the transformed fungi in field studies. These studies, to generate genetically marked yet effective biocontrol agents for fire ant control are important so as to monitor the efficacy of the introduced organism in the field and to assess the impact on beneficial species of insects.

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LITERATURE CITED

Bextine, B. 1998. Application of *Beauveria bassiana* alginate pellets as a biological control strategy against *Solenopsis invicta*. M.S. thesis, Texas Tech University, Lubbock. 113 p.

Broome, J. 1974. Microbial control of the imported fire ant, *Solenopsis richteri* (Forel) (Hymenoptera: Formicidae). Ph. D. dissertation. Mississippi State University, State College.

Couteaudier, Y., M. Daboussi, A. Eparvier, T. langin and J. Orcival. 1993. The GUS gene fusion system (*Escherichia coli* b-glucuronidase gene) a useful tool in studies of root colonization by Fusarium oxysporium . Appl. Environ. Microbiol. 59: 1767-1773.

Jefferson, R. A., T. A. Kavanagh, and M. W. Bevan. 1982. GUS fusions-β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. 6: 3901-3907.

Knudsen, G, J. Johnson, and D. Eschen. 1990. Alginate pellet formulation of a *Beauveria bassiana* (Fungi: Hyphomycetes) isolate pathogenic to cereal aphids. J. Econ. Entomol. 83: 2225-2228.

Lofgren, C.S., and D. F. Williams. 1982. Avermectin B_1a: Highly potent inhibitor of reproduction by queens of the red imported fire ant (Hymenoptera: Formicidae). J. Econ. Entomol. 75: 798-803.

McNeely, W., and D. Pettitt. 1973. Algin. Pp. 49-8. *In* Whistler, R. (ed.). Industrial gums. Polysaccharides and their derivatives. 2nd ed. Academic Press, NY. 807 pp.

Oi, D., R. Periera, J. Stimac, and L. Wood. 1994. Field applications of *Beauveria bassiana* for control of the red imported fire ant (Hymenoptera: Formicidae). J. Econ. Entomol. 87: 623-630.

Pfeifer, T. A., and G. G. Khachatourians. 1992. *Beauuveria bassiana* protoplast regeneration and transformation using electroporation. Appl. Microbiol. Biotechnol. 38:376-381.

Quattlebaum, E. 1980. Evaluation of fungal and nematode pathogens to control the red imported fire ant, *Solenopsis invicta* Buren. Ph. D. dissertation. Clemson University, Clemson, SC.

Sambrook, J., E. F. Fritsch and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, NY. 809 pp.

Sanchez-Pena, S. 1992. Entomopathogenic fungi against the red imported fire ant, *Solenopsis invicta* Buren (Hymenoptera: Formicidae). M.S. thesis. Texas Tech University, Lubbock.

Siebeneicher, S. R., S. B. Vinson, and C. M. Kenerley. 1992. Infection of the red imported fire ant by *Beauveria bassiana* through various routes of exposure. J. Invertebr. Pathol. 59: 280-285.

Stimac, J., R. Pereira, S. Alves, and L. Wood. 1993. *Beauveria bassiana* (Balsamo) Vuillemin (Deuteromycetes) applied to laboratory colonies of *Solenopsis invicta* Buren (Hymenoptera: Formicidae) in soil. J. Econ. Entomol. 86: 348-352.

Walker, H. and W. Connick, Jr. 1983. Sodium alginate for production and formulation of mycoherbicides. Weed Sci. 31: 333-338.

White H. 1995. Alginate pellet formulation of *Beauveria bassiana* pathogenic to the red imported fire ant. M.S. thesis. Texas Tech University, Lubbock.