



Texas Imported Fire Ant Research and Management Project

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Beauveria bassiana as a Biocontrol Agent Against the Red Imported Fire Ant

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Major Accomplishments to date:

1. Genetic Manipulations. The fungal transformation expression vector, pBARGPE1 was provided by Dr. Krishna Podila, Michigan Technological University. The vector had the *Aspergillus nidulans gpdA* promoter and the *A. nidulans trpC* terminator flanking a multiple cloning site. The β -glucuronidase gene (GUS) was 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 *Bam*H1-*Eco*R1 fragment. The *Bam*H1 site was blunt-ended using T4 DNA polymerase. Transformation vector pBARGPE1 was digested with *Xho*1, blunt-ended, and digested with *Eco*R1. The GUS gene was thus directionally cloned under control of the *gpdA* promoter using T4 DNA ligase. The resulting 7.3 kb plasmid construct was transformed into *Escherichia coli* DH5 using CaCl_2 transformation.

2. Spheroplast Production. *Beauveria bassiana* was grown on Sabourand dextrose agar (SDA) for 8-10 days 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 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 mycelia. Blastospores were harvested by centrifugation using a Sorvall 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 was 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 hour 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 KH₂PO₄, 600 mM KCl (pH 5.8). Cell wall-degrading enzymes [chitinase (0.5% w/v), cellulase (1% w/v), lysozyme (1% w/v), and α -glucuronidase (0.5% v/v)] were added to generate osmotically stable spheroplasts that were incubated unshaken for 3 hours at 30°C. Spheroplasts were then washed with 0.6 M sorbitol twice at 5 k rpm for 10 minutes at 4°C. The spheroplasts were finally re-suspended to 10⁸ cells/ml in 0.6M sorbitol and stored on ice until use.

3. Electroporation of Spheroplasts. The electroporation protocol was a modification of that described by Pfeifer and Khachatourians (1992). Five μ g of linear plasmid DNA, pBARGPE1-GUS, was added to a 200- μ l 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 μ F 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 NH₄SO₄ added as a stabilizer and incubated for 2 hours 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 hours of growth, the plates are over-laid with YP agar containing 700 μ g/ml of BASTA for selection and incubated at 30°C until transformants appeared.

4. α -glucuronidase Assay. Transformants were sub-cultured onto YP-BASTA plates. Once these cultures were established, 1 cm² 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 NaPO₄ [pH 7.0], 10 mM β -mercaptoethanol, 10 mM EDTA, 0.1% Triton X-100) and incubated at 37°C for 20 minutes. Following this, 5 μ l of 0.5 mM potassium ferricyanide, 5 μ l 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.

5. Generation of a Genetically Tagged Strain of *B. bassiana*. The α -glucuronidase gene from *E. coli* was successfully cloned into the fungal vector pBARGPE1 to generate the plasmid pBARGPE1-GUS using restriction enzymes *NotI* and *EcoRI*. This construct was used to transform *B. bassiana*.

6. Transformation of *B. bassiana*. Spheroplasts were derived from germinating mycelia, 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.

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°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 those isolates (7A, 7B, 13A, and 13B) that retained the ability to grow on media containing the anti-fungal antibiotic BASTA were chosen. The GUS transformants, the wild type strain, and *E. coli* were all grown under appropriate conditions, protein extracts were isolated in lysis buffer, and fluorescence was measured. 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. 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 show the presence of the GUS gene in the fungal genome. Positive hybridization with the GUS gene probe, as indicated by autoradiography, was observed with the *B. bassiana* transformants and the positive control, but not with the wild type, untransformed strain. In the transformants, the GUS gene probably existed only as single or few copies, and this could explain the less abundant signal.

7. Bioassays. Initial studies using the *B. bassiana* GUS transformants indicated that the transformants are effective in killing *S. invicta* in the laboratory environment. Over the assay period of one week, however, we observed that the parental strain of the fungus was more efficient in killing the ants; the mortality rate was slightly higher than with the transformed isolates. The wild type fungus killed 85% of the ants in the first three days of the assay. Two of the tested transformants killed 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 died over the first four days, and only 25% were dead after one week. The differential capabilities of the transformants to kill *S. invicta* vary depending upon the site at which the GUS gene is inserted into the fungal genome. Thus it is vital that laboratory assays be carried out before field trials commence. 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 *S. invicta*, are important so as to monitor the efficacy of the introduced organism in the field and to assess the impact on beneficial insect species.

Agreements. Agreements have been made between the principal investigators and Cook, T., F. Mitchell, S. B. Vinson, and J. Fuxa ("Biological control of the RIFA through American augmentation utilizing native diseases"), also supported by the Texas Fire Ant Initiative Program. The interactions will include expanded opportunities for laboratory and field trials.

Goals Achieved:

Beauveria bassiana has been transformed by addition of genes that will assist in laboratory procedures (Benomyl resistance), will identify our strains (GUS marker genes), and will augment infectivity and pathogenicity (future work, chitinases) of our fungal strain. Transformed strains grow well in broth culture, and cause mortality of *Solenopsis invicta* in laboratory bioassays. Also, our delivery system of alginate pellets of *B. bassiana* is acceptable to *S. invicta* foragers.

Relevance to the Texas Imported Fire Ant Research and Management Project:

Our strains and formulations of *B. bassiana* cause mortality of *S. invicta* in laboratory and field trials. *Beauveria bassiana* may be especially useful biological control agent in management of *S. invicta* populations in environmentally sensitive habitats such as near waterways, in recreational areas, and where threatened or endangered animals species are present.

Publications, Citations, Paper Citations, and other Citable Products:

Anderson, J., H. Thorvilson, and S. Russell. 2001. Natural landscape materials as repellents of red imported fire ants. Southwest. Entomol. (accepted)

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Bextine, B., and H. Thorvilson. 2001. Field applications of bait-formulated *Beauveria bassiana* alginate pellets as a biological control of the red imported fire ants (*Solenopsis invicta*). Environ. Entomol. (submitted)

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Thorvilson, H., and M. San Francisco. 2001. Development of *Beauveria bassiana* formulations and genetically marked strains as a potential biopesticide for imported fire ant control. Symposium Presentation, 2001 Imported Fire Ant Conference, San Antonio, Texas, 28 February 2001.

Thorvilson, H., D. Wheeler, B. Bextine, and M. San Francisco. 2001. Development of *Beauveria bassiana* formulations and genetically marked strains as a potential biopesticide for imported fire ant control. Southwest. Entomol. (accepted).

Wheeler, D., M. San Francisco, and H. Thorvilson. 2001. Engineered *Beauveria bassiana* for field identification of insect mortality. Mycologia (in preparation)