

**2006 GSCSSA TERMINATION REPORT**  
for  
**AWARD G001579**  
(Sept. 15, 2003 to Sept. 14, 2006)

**TITLE OF PROJECT: Control of Grassy Weeds. Development of a Novel, Highly Specific, Naturally-Occurring Bioherbicide.**

**OBJECTIVES:**

- A. Determination of the chemical structure of the bioherbicide.**
- B. Development of methods for the production of the bioherbicide at scales sufficient for greenhouse and/or field plot testing**
- C. Demonstration of the efficacy of the bioherbicide in greenhouse and/or small-scale field plot trials.**
- D. Protection of the research with U.S. and international patents.**

**INVESTIGATORS: Donald Armstrong and Dallice Mills**

Department of Botany & Plant Pathology  
Oregon State University  
Corvallis, OR 97331-2902  
Email: armstrod@science.oregonstate.edu,  
millsd@science.oregonstate.edu  
Phone: DA (541)-737-5291; DM (541)-737-5303

**COOPERATOR: Mark Azevedo (USDA-ARS, NFSPRC, Corvallis, OR)**

**ABSTRACT OF RESULTS:** We have demonstrated that certain isolates of rhizosphere bacteria produce a Germination-Arrest Factor (GAF) that specifically arrests germination of the seeds of a wide-range of grassy weeds. The biological properties of GAF, the compound's physical and chemical characteristics, and the identity of certain genetic regulatory elements that control GAF production have been established. We have also succeeded in purifying GAF to homogeneity and in obtaining mass spectral and nuclear magnetic resonance data that have enabled us to assign a putative structure to the active compound. A U.S. patent application has been filed.

**JUSTIFICATION:** Contamination of perennial ryegrass and tall fescue seed lots with the seeds of a variety of grassy weeds is a major problem to the grass seed industry in the Pacific Northwest. This problem is made worse by the development of Diuron-resistant strains of these weeds. We have identified a naturally occurring herbicide that selectively arrests germination of the seeds of a wide range of grassy weeds, including Diuron-resistant strains. Commercial development of this herbicide will provide a highly specific and effective alternative to synthetic chemical herbicides and one that is likely to have a minimal impact on the environment.

## **ACCOMPLISHMENTS:**

**Summary of Major Project Accomplishments.** We have previously described the isolation and characterization of 5 bacterial strains that produce and secrete a novel herbicidally-active compound that we have termed a Germination Arrest Factor (GAF). These bacterial isolates were obtained from soils of the Willamette Valley and were identified as particular strains of *Pseudomonas* species. The compound (GAF) produced by these bacteria specifically arrests the germination of a wide range of grassy weeds, including annual bluegrass, jointed goatgrass, Diuron-resistant strains of downey brome and rattail fescue, tall fescue, domestic and wild oats, Ventenata grass, and perennial ryegrass, while exerting little if any effect on the post-germination growth of grass seedlings or germination of the seeds of a number dicot (broad-leaf) species. Three bacterial genes that regulate GAF production have been identified and sequenced. Mutational analysis of these genes demonstrated that GAF activity was associated with a specific ninhydrin-reactive compound that failed to partition into organic solvents or bind to reverse phase chromatographic materials. The very hydrophilic character of GAF posed major problems in its purification, but we were eventually successful in obtaining purified GAF preparations that have permitted mass spectral and nuclear magnetic resonance analysis of its structure. The data obtained from these studies have permitted us to assign a putative structure to the active compound. This result has created the opportunity to design a chemical synthesis of GAF, first to confirm the putative structure of the molecule and subsequently to attempt production of the compound in quantities sufficient for field testing. In the interim, we have used partially-purified GAF preparations to demonstrate that GAF is active in inhibiting weed-seed germination in Willamette Valley soils as tested under small scale laboratory and growth chamber conditions.

### **Objective A. Determination of the Chemical Structure of the Bioherbicide.**

Establishing the structure of GAF is essential to the commercial development of this compound as an herbicide. Knowledge of the precise structure of the compound will facilitate patent description, provide strategies for the commercial production of the compound, and encourage interest on the part of members of the agricultural chemicals industry. Therefore, determination of the chemical structure GAF has been a major focus of our research efforts.

The initial problem that had to be overcome for the structural characterization of GAF was the development of methods for its purification. GAF is a highly water-soluble (hydrophilic) compound. This property should be useful for purposes of herbicidal formulation, but it greatly complicates purification because of the inability to use either conventional solvent extraction procedures or chromatographic and trace enrichment methods based on hydrophobic interactions to effect separation of GAF from contaminating compounds. An initial partial purification of GAF from bacterial culture filtrates was achieved by procedures involving extraction of dried culture filtrates with aqueous ethanol solutions under carefully defined conditions, followed by thin-layer chromatography (TLC) of the resulting extracts on cellulose TLC plates. These procedures, in combination with mutational analysis achieved by transposon mutagenesis of one of the GAF-producing strains of bacteria, enabled us to

demonstrate that GAF activity was associated with a specific ninhydrin-positive compound present in culture filtrates derived from the bacterial strains that produce GAF and to determine a number of the chemical and physical properties of the GAF molecule. Based on these results, preparative TLC separations were used to obtain GAF preparations that were essentially free of other ninhydrin-reactive organic compounds and contained reduced levels of the inorganic salts present in the culture filtrates. Although preparative TLC methods are very labor intensive, these partially purified GAF preparations had to serve as the starting point for all subsequent purification efforts. Achieving the rigorous purification of GAF required for structural analysis proved extremely difficult, and trials of a number of strategies were necessary before success was eventually obtained. In 2005, we were able to identify a property of the GAF molecule that greatly facilitated chromatographic work with this compound, and this discovery enabled us to develop a method for the purification of the unmodified GAF compound in quantities sufficient for rigorous chemical analysis.

Mass spectral studies of samples of the purified GAF compound were performed with the aid of the OSU Mass Spectrometry Facility. These studies yielded molecular weight data, relatively complete fragmentation patterns, and high resolution mass values for the GAF molecule. The latter data enabled us to generate a list of possible empirical formulas, of which one seemed most likely on the basis of the observed fragmentation patterns. This conclusion has subsequently been confirmed by NMR studies carried out in collaboration with Dr. Kerry McPhail of the College of Pharmacy. The NMR studies have also provided sufficient data concerning the functional groups present in the molecule and their relationship to each other that Dr. McPhail was able to assign a putative structural formula to the GAF molecule. The analytical results appear relatively unambiguous, except for assignment of the correct stereoisomer configurations associated with the structure (*i.e.* essentially a determination of whether we are dealing with right-handed or left-handed versions of the GAF molecule). Resolution of these remaining structural details, as well as final confirmation of the putative GAF structure, requires chemical synthesis of GAF. This work is currently in progress.

**Objective B. Development of methods for the production of the bioherbicide at scales sufficient for greenhouse and/or field plot testing.** The development of methods for the large scale production of GAF is likely to be dependent on achieving a cost-effective chemical synthesis. Work on the small-scale chemical synthesis of GAF for structural confirmation and demonstration of concept for larger scale work is ongoing. Because it is possible that a combination of chemical and enzymatic methods may be the most effective way to proceed in the future, we are also interested in elucidating the GAF biosynthetic pathway. To this end, as time and resources permit, we have been attempting to identify the genes that code for the enzymes directly involved in the biosynthesis of GAF.

**Objective C. Demonstration of the efficacy of the bioherbicide in greenhouse and/or small-scale field plot trials.** We have been able to demonstrate the efficacy of partially purified preparations of GAF in arresting the germination of

seeds of grassy weeds in soil-based systems in laboratory, growth chamber, and greenhouse tests. Moreover, in greenhouse tests of established seedlings of tall fescue, perennial rye grass, and wheat, GAF spray treatments were without statistically significant effects on the post-germination growth of these crop species. Based on these results, we are encouraged to believe that GAF spray treatments can be used to suppress the germination of weed seeds in perennial grass cropping systems.

**Objective D. Protection of the research with U.S. and international patents.**

To develop patent protection for GAF and the GAF-producing bacterial strains, we have worked with the OSU Technology Transfer Office, with Margaret Connor (the USDA Patent Advisor), and with attorneys at Klarquist Sparkman who handle patentable inventions. A provisional USA patent application was filed in December, 2002, and patent protection was subsequently extended by filing a Patent Cooperative Treaty International Application in December, 2003. In December, 2004, a decision was reached to limit our filings under the Cooperative Treaty to a provisional US-patent application. (Cost considerations have prohibited the University from exercising the option of additional filings in international jurisdictions.)

**TIMELINE FOR ACCOMPLISHMENTS REPORTED HERE:**

**Objective A:** Purified samples of unmodified GAF were obtained in amounts sufficient to initiate mass spectral analysis in May 2005. Mass spectral data, including fragmentation patterns and high resolution mass estimates, were obtained during the summer of 2005. Nuclear magnetic resonance (NMR) studies of purified GAF samples were initiated in September 2005, and a putative structural formula was assigned to the GAF molecule by the first week of October.

**Objective B:** Methods for the large-scale production of GAF are still under development at this writing. Demonstration scale chemical synthesis approaches are in progress, and biochemical information that may aid in cost-effective synthesis is being developed.

**Objective C:** Initial tests of the efficacy of partially purified GAF preparations in spray applications to soil systems in growth chamber and greenhouse environments were completed in June, 2004.

**Objective D:** Our work is currently protected under a modified provisional U.S. patent protection application filed in December 2004.

**PUBLICATIONS, REPORTS, AND PRESENTATIONS FOR GRANT PERIOD:**

Journal publications have not been submitted to date because of patent protection concerns, but three manuscripts are in draft form. The first public presentation of results obtained in studies of GAF occurred at the GSCSSA Meeting in Portland, Oregon in December, 2002. Results of our initial efforts to purify GAF were presented at the Portland GSCSSA Meeting in November of 2003, and progress reports were presented at the GSCSSA Meetings in Moscow, Idaho, and Albany, Oregon in November 2004 and November 2005 respectively.