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Germination-Arrest Factor (GAF): 3. Determination that the herbicidal activity of GAF is associated with a ninhydrin-reactive compound and counteracted by selected amino acids ☆

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ABSTRACT

A novel, naturally-occurring herbicide (Germination-Arrest Factor, GAF), produced by *Pseudomonas fluorescens* WH6 and several related isolates of rhizosphere bacteria, irreversibly arrests germination of the seeds of a wide range of graminaceous species, including a number of important grassy weed species. GAF activity has been shown previously to be associated with a hydrophilic, low molecular weight compound that contains an acid group. In the present study, thin-layer chromatography (TLC) of extracts of WH6 culture filtrate demonstrated that GAF activity migrates on TLC plates with a particular ninhydrin-reactive compound. This compound was found to be present in GAF-producing *P. fluorescens* isolates and absent in *P. fluorescens* strains that lack the ability to produce GAF. Treatments, including mutagenesis, which resulted in the loss of GAF activity in culture filtrates from *P. fluorescens* WH6 were shown to result in the disappearance of this ninhydrin-reactive compound from extracts of WH6 culture filtrates or in alteration of its appearance on TLC chromatograms. The ninhydrin-reactivity of GAF indicates that it probably contains an amino group, as well as the acid group previously demonstrated, and suggests that GAF may be a small peptide or amino acid analog. Biological investigations motivated by this conclusion demonstrated that the effects of GAF in inhibiting the germination of seeds of annual bluegrass (*Poa annua* L.) could be counteracted by treatment with alanine or glutamine and, to lesser extent, by several other amino acids, suggesting that this compound may act by interfering with some aspect of amino acid metabolism or function.

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1. Introduction

Rhizosphere bacteria that exert inhibitory effects on the growth and development of various higher plant species have been isolated from the soil environment by a number of investigators (Nehl et al., 1996). The potential of such bacteria (Deleterious Rhizosphere Bacteria, DRB) to serve as agents for the biocontrol of weed species has received considerable attention, but met with limited practical success to date. Many DRB have been identified as *Pseudomonas* species, and pseudomonads are known to produce a wide range of secondary products, including a number of compounds that have antimicrobial and/or antifungal activity (Lesinger and Margraff, 1979; Dowling and O'Gara, 1994). However, in most cases, the compounds responsible for the effects of these organisms on higher plant species have yet to be identified.

The development of biological or chemical agents for the control of grassy weeds in the grass seed production systems that constitute an important element of agricultural activity in the Pacific Northwest has been a focus of our investigations. To this end, we have been interested in identifying DRB that specifically target germination of the seeds of grassy weeds and in characterizing the biologically active compounds produced by such DRB. We have previously described the isolation and characterization of five strains of rhizosphere bacteria that produce and secrete a novel, naturally-occurring herbicide that irreversibly arrests the germination of seeds of a large number of graminaceous species, including those of a number of important grassy weeds (Banowetz et al., 2008). These bacterial isolates were identified as strains of *Pseudomonas fluorescens.* The herbicide produced by these strains arrests seed germination in a developmentally-specific manner, halting the germination process immediately after the emergence of the plumule and coleorhiza. Because germination is initiated

☆ The first paper in this series was Banowetz et al. (2008).

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in the presence of the herbicide and then subsequently arrested, we have described this compound as a Germination-Arrest Factor (GAF) rather than a germination inhibitor. The effects of GAF were found to be limited primarily to germination, with little effect observed on the growth of established seedlings or mature plants. Moreover, the seeds of dicot species were shown to be less sensitive to GAF than seeds of graminaceous species. A quantitative bioassay for GAF activity was developed (Banowetz et al., 2008) using seeds of annual bluegrass (ABG, *Poa annua* L.), a grassy weed of economic significance in grass seed production.

An initial characterization of the physical and chemical properties of the GAF compound produced by one of our isolates, *P. fluorescens* WH6, was reported in a previous publication (Banowetz et al., 2009). GAF activity was found to be associated with a small (molecular weight less than 1000), hydrophilic compound that contained an acid group. GAF activity could be recovered from dried culture filtrates by extraction with appropriate concentrations of aqueous ethanol, but it was essentially insoluble in other organic solvents with the exception of methanol, in which it was moderately soluble. GAF activity was found to be destroyed by heating at temperatures above 65 °C, and there was a significant loss of activity in sterile WH6 culture filtrates after storage for several months at 4 °C.

The chemical and chromatographic properties of GAF have been further examined in the current study. We present evidence here that GAF activity is associated with a particular ninhydrin-reactive compound that can be recovered from WH6 culture filtrate solids by extraction with aqueous ethanol solutions. This result indicates that GAF is likely to contain an amino group as well as the acid group detected earlier. In addition, the inhibitory effects of GAF on seed germination were found to be reversed in the presence of particular amino acids. These results, taken together and with our earlier data, suggest that the compound responsible for GAF activity may be a small peptide or an amino acid analog.

2. Materials and methods

2.1. Biological materials

The isolation and characterization of the GAF-producing isolates of *P. fluorescens* used here (Isolates WH6, AD31, AH4, E34, and WH19) have been described previously (Banowetz et al., 2008). The strains of *P. fluorescens* used as controls were obtained as follows: *P. fluorescens* Pf-5 (Howell and Stipanovic, 1979; Loper and Gross, 2007) and *P. fluorescens* A506 (Wilson and Lindow, 1993) were obtained from Dr. Joyce Loper (USDA-ARS Horticultural Crops Research Laboratory, Corvallis, OR). *P. fluorescens* PfO-1 (Compeau et al., 1988) was kindly provided by Dr. Mark Silby (Department of Molecular Biology and Microbiology, Tufts University School of Medicine, Boston, MA). *P. fluorescens* D7 (NRRL B-18293) (Gurusiddaiah et al., 1994; Kennedy et al., 1991, 2001) was obtained from Dr. Ann Kennedy (USDA-ARS, Pullman, WA). The transposon vector pUTmini-Tn*5gfp* (Tn*5gfp*), in its host *Escherichia coli* S17-1 λ *pir* (Matthysse et al., 1996), was obtained from the American Type Culture Collection (ATCC 87454) and as a gift from A. Matthysse.

Annual bluegrass seeds (ABG, *Poa annua* L.) were obtained from 1996 mid-Willamette Valley grass seed screenings and were provided by International Seeds, Halsey, OR, and C and R Farm, Tangent, OR. The seeds were cleaned to remove straw and seeds of other species prior to use.

2.2. Solvents

All aqueous ethanol solutions were prepared from 95% (v/v) ethanol that had been redistilled before use. All other solvents were purchased as spectrophotometric grade reagents.

2.3. Growth of bacterial cultures and preparation of bacterial culture filtrates

P. fluorescens WH6 and the other isolates and strains of *P. fluorescens* used in investigations of GAF activity were taken from cryovial storage in 50% glycerol at −60 °C and inoculated into Wheaton bottles half-filled with sterile *Pseudomonas* Minimal Salts Medium (PMS). The PMS Medium was modified from that described by Bolton et al. (1989) by supplementation with iron as described by Banowetz et al. (2008). The tops of the bottles were loosely capped and secured with tape. The inoculated bottles were placed on a rotary shaker (200 rpm) in a 27 °C chamber. Cells were harvested after 7 days in culture.

To prepare culture filtrates, the *Pseudomonas* culture fluid recovered from 7-day cultures was centrifuged (3000 *g*, 15 min), and the supernatant was passed through a bacteriological filter (Millipore GP Express Steritop, 0.22 μm pore size). The resulting sterile culture filtrate was stored at 4 °C.

For molecular genetic studies, *P. fluorescens* WH6 was maintained on solid Fluorescent *Pseudomonas* Medium (FPM) (Sands and Rovira, 1970; Simon et al., 1973) at room temperature and grown in Luria broth (LB) medium at 28 °C for transformation with pUTmini-Tn*5gfp*. *E. coli* were grown in LB medium at 37 °C (Sambrook and Russell, 2001). All solid media contain 1.5 percent Difco Bacto-Agar (Difco Laboratories, Detroit, MI). Tetracycline (15 μg mL^{-1}) and ampicillin (50 μg mL^{-1}) were added when appropriate for selection.

2.4. Extraction of culture filtrate solids with 90% (v/v) ethanol

Measured volumes of bacterial culture filtrate were taken to dryness *in vacuo* at a temperature ⩽45 °C. The dry solids remaining after evaporation of the filtrate were extracted three times (5 min per extraction) with 90% (v/v) ethanol. Each of these three extractions was performed by swirling the solids with a volume of solvent equal to one-third of the original volume of culture filtrate. The three extracts prepared in this manner were combined, taken to dryness *in vacuo* as described above, dissolved in a volume of 76% (v/v) ethanol equal to one-twentieth of the original volume of culture filtrate (20× concentration), and either applied immediately to thin-layer chromatography (TLC) plates (as described below) or stored at 4 °C or later use.

2.5. Bioassay of GAF activity

Bioassays for GAF activity were performed with ABG seeds using the standard GAF bioassay protocol described by Banowetz et al. (2008). Culture filtrates and other solutions to be tested for GAF activity were distributed to the wells of sterile 48-well plates (Corning Costar 3548). Each well received 200 μL of sterile test solution and three ABG seeds from seed lots that had been surface-sterilized according to the procedure described in Banowetz et al. (2008). Controls consisted of seeds incubated in sterile water or sterile, un-inoculated PMS medium. Both control treatments were without effect on the germination of ABG seeds. Three replicate wells (9 seeds) were prepared for each concentration of each treatment tested. The plates were sealed with Parafilm and incubated in a growth chamber at 20 °C with a photoperiod of 8 h light (50 μmol m^{-2} s^{-1}) and 16 h dark. Germination scores were determined after 7 d incubations.

The germination scoring system used to evaluate GAF bioassays has been described in detail by Banowetz et al. (2008). In an abbreviated outline, germination scores in this system range from 1 to 4, with a score of 4 representing normal development (no GAF activity) at the end of the 7-day assay period. A score of 1 is assigned to seeds where the plumule and coleorhiza have emerged from the