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

Donald Armstronga, Mark Azevedob, Dallice Millsa, Bonnie Baileya, Brian Russella, Aleta Groeniga, Anne Halgrenb, Gary Banowetzb,*, Kerry McPhaillc

aDepartment of Botany and Plant Pathology, Oregon State University, Corvallis, OR 97331, United States
bUSDA-ARS, NFPSC National Forage Seed Production Research Center, Corvallis, OR 97331, United States
cCollege of Pharmacy, Oregon State University, Corvallis, OR 97331, United States

A B S T R A C T

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.

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
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-Tn5gfp (Tn5gfp), in its host Escherichia coli Si7-1 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-Tn5gfp. 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 (<5 μl) and plates were 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
seed but both are shorter than the length of the seed. A score of 2 indicates the primary root is visible and the plumule is slightly longer than the seed, but typically chlorotic. A score of 3 indicates that the first true leaf has emerged from the coleoptile and is green in color, but the emerged portion of the leaf is obviously shorter than the length of the coleoptile. A score of 4 is assigned to seeds exhibiting normal development, equivalent to that of controls, which at this stage of germination means that the first true leaf is fully emerged, green in color, and the emerged portion of the leaf is obviously longer than the coleoptile. A score of zero (no visible evidence of seed germination) is possible in this system, but GAF concentrations equivalent to that of full-strength WH6 culture filtrate typically result in permanent arrest of development at a germination score of 1.

Quantitative estimates of the relative amounts of GAF present in various extracts are expressed in terms of GAF milliequivalents, where one GAF milliequivalent is defined as the minimum quantity of GAF required to give a germination score of 1 when dissolved in 1 mL of bioassay test solution. GAF milliequivalents were determined from a standard graph prepared by plotting germination score against various dilutions of a WH6 culture filtrate. (For an illustration of such a graph, see Banowetz et al., 2009.)

2.6. Amino acid and GAF competition experiments

Amino acid stock solutions were prepared at concentrations two-fold higher than those to be tested and adjusted to pH 6.5 with HCl or NaOH. The resulting solutions were filter-sterilized (0.2 μM filter). Similarly, sterile culture filtrate from P. fluorescens WH6 was diluted to a concentration two-fold higher than that to be tested. Equal volumes (100 μL each) of the appropriate amino acid and culture filtrate stock solutions were then mixed and distributed to assay plate wells for testing in the standard GAF bioassay system as described above.

2.7. Thin-layer chromatography (TLC) materials and procedures

TLC separations were performed on Avicel® Microcrystalline Cellulose Uniplates (5 × 20 cm, 250 μm layer, glass-backed) and on Hard-Layer Silica Gel GHL Uniplates (5 × 20 cm, 250 μm layer, glass-backed, with an inorganic binder), both purchased from Analtech, Inc. The TLC chromatograms were developed in cylindrical chromatography tanks (6 × 23 cm) containing 25 mL of solvent. For chromatography on the Avicel cellulose TLC plates, the chromatographic solvent consisted of ethyl acetate:isopropanol:water (7.5:15:10). For chromatography on the silica gel GHL plates, the solvent system consisted of ethyl acetate:isopropanol:methanol:water (5:5:18:2).

The chromatographic samples consisted of 200 μL aliquots of the 76% ethanol extracts (20× solutions) prepared from 90% ethanol of dried culture filtrates as described above. The 200 μL aliquots of the extracts to be analyzed were each streaked across a TLC plate by repeated applications to an origin line located 3 cm from one end of the plate. The sample was applied with a 50 μL Lang–Levy micropipette equipped with a screw-type micropipette-control device and using a filtered air-stream to dry the origin for 20–30 s between applications. Following the final application, the chromatograms were allowed to air dry for 4 or 8 min (silica gel or cellulose plates, respectively) and then developed over a distance of 12 cm from the origin.

For ninhydrin-staining of developed chromatograms, the dry chromatograms were sprayed with a ninhydrin solution consisting of 0.25% (w/v) ninhydrin (Sigma) dissolved in 95% (v/v) ethanol containing 3.0 mL of glacial acetic acid per 100 mL of solution. Color development was achieved by heating the sprayed chromatograms in an oven at 80–90 °C for 15 min.

For bioassay of the distribution of GAF activity on cellulose TLC chromatograms, the cellulose was scraped from developed TLC plates as 1 cm bands located between the origin and solvent front. The cellulose from each zone (1 × 5 cm) was suspended and vortexed in 1.0 mL of water in a 2.0 mL Minifuge tube. The cellulose was then pelleted by centrifugation (15,000 rpm, 10 min, Mini-fuge). The supernatant from each tube was filter-sterilized (0.2 μm filter), diluted to an appropriate concentration with sterile water, and tested for GAF activity in the standard GAF bioassay system as described above.

2.8. Transposon mutagenesis of P. fluorescens WH6

The plasmid pUTmini-Tn5gfp was extracted from E. coli S1701 cells using standard procedures (Sambrook and Russell, 2001). Overnight cultures of P. fluorescens WH6 cells grown in 20 mL of LB at 28 °C with shaking (200 rpm) were centrifuged at 3330g for ten minutes and washed. The cell pellets were cooled on ice, suspended in 1 mL of sterile double deionized water, and 600 μL ice-cold LB was then added to each. Cell suspensions were transferred to electroporation cuvettes and transformed by pUT-mini-Tn5gfp using a BTX Electro Cell Manipulator 600 (Harvard Apparatus, Inc., Holliston, MA) according to the manufacturer’s protocol for P. putida transformation. Both a non-transformed control and the electroporated cell suspension were transferred to ice-cold, sterile 50 mL centrifuge tubes, which were then incubated on a shaker (200 rpm) at 28 °C for 17 h. A library of TetR colonies was selected on solid LB medium containing tetracycline. These colonies were inoculated into PMS medium for the production of culture filtrates, which were subsequently screened for GAF activity in the standard GAF bioassay system described above.

3. Results

3.1. TLC analysis of the GAF activity in extracts of P. fluorescens WH6 culture filtrate

The hydrophilic character of the compound responsible for GAF activity limits options for its purification. To achieve an initial separation of GAF from some of the salts in the culture filtrate, the solids recovered after evaporation of culture filtrate from the GAF-producing isolate P. fluorescens WH6 were extracted with 90% ethanol as described in Section 2. As shown previously (Banowetz et al., 2009), this procedure leaves behind 30–40% of the GAF activity, but the resulting extract is more amenable to fractionation procedures than the culture filtrate itself and proved to be a useful starting point for subsequent fractionation and analysis of GAF activity.

The GAF activity recovered from the WH6 culture filtrate solids by extraction with 90% ethanol was examined by TLC fractionation. As shown in Fig. 1, when these extracts were chromatographed on microcrystalline cellulose TLC plates using an appropriate solvent system, GAF activity could be recovered from the chromatograms as a single broad band of activity in a zone located in the lower half of the plate (Fig. 1A). This zone contained no obvious UV-absorbing or fluorescent compounds when examined under UV-light. Tests of various color reagents revealed that this band of GAF activity was coincident with a zone on the chromatograms that gave a strongly positive color reaction when the chromatograms were sprayed with ninhydrin (Fig. 1B).

3.2. TLC comparisons of GAF-producing and non-producing strains of P. fluorescens

To determine whether the ninhydrin-positive band that co-chromatographed with GAF activity in TLC fractions of
The association of GAF activity with the particular ninhydrin-reactive compound identified above was confirmed by mutagen-
sis of P. fluorescens WH6. Transposon mutagenesis of WH6 with the Tn5 transposon coding for tetracycline resistance resulted in the generation of 1,214 tetracycline-resistant transformants. Screening of culture filtrates from these transformants in the standard GAF bioassay system resulted in the identification of three transformants (WH6-1::Tn5, WH6-2::Tn5, and WH6-3::Tn5) that appeared to have lost the ability to produce GAF. The apparent loss of GAF activity in the WH6-1::Tn5 mutant proved to be difficult to reproduce, and additional work indicated that this mutation had affected growth characteristics rather than GAF production. Culture filtrates from the other two mutants (WH6-2::Tn5 and WH6-3::Tn5), however, were confirmed to lack GAF activity. As shown in Table 2, the GAF activity in culture filtrates from these two mutants was essentially eliminated. TLC analyses of the culture filtrates from WH6-2::Tn5 and WH6-3::Tn5 are shown in Fig. 4. The prominent ninhydrin-positive band visible on the TLC plate generated from wild type WH6 culture filtrate was absent from both the WH6-2 and WH6-3 culture filtrates. Thus, mutagenic loss of GAF activity also resulted in loss of the particular ninhydrin-reactive compound present in culture filtrates of this GAF-producing line of P. fluorescens.

3.5. Influence of amino acids on GAF activity in the Poa bioassay

The ninhydrin-reactivity of GAF indicates that it probably contains an amino group. This fact, together with our previous demonstration that GAF is a low molecular weight compound that contains an acid group (Banowetz et al., 2009) suggested that GAF might be some type of small peptide or an amino acid analog. The latter hypothesis led us to suspect that it might be possible to counteract the effects of GAF by treatment with particular amino acids. To explore this possibility, the effects of the twenty protein amino acids on the response of ABG seeds to WH6 culture filtrate were examined in the standard GAF bioassay system. For purposes of testing this interaction, the WH6 culture filtrate concentration used in the bioassay was reduced to the minimal level that would cause complete, or nearly complete, germination arrest (about 0.1 \( \times \) in this case), and the response of ABG seeds to this filtrate concentration was examined in the presence of varying concentrations of the test amino acid. The results are summarized in Table 3.

Three amino acids were found to significantly alter the response of ABG seeds to GAF under the described test conditions. The effects observed with alanine and glutamine were particularly dramatic. At appropriate concentrations, both of these amino acids were able to completely counteract the effects of the 0.1 \( \times \) culture filtrate on ABG germination (yielding bioassay scores of 4), with alanine being the most active of the two. As shown in Fig. 5, alanine was still able to counteract the effects of GAF when the concentration of the latter was increased two- to four-fold. Phenylalanine, tyrosine, leucine, serine, methionine, isoleucine, and glutamic acid also significantly counteracted the effects of GAF. With the exception of tyrosine, all yielded germination scores of 3 or greater in the presence of 0.1 \( \times \) culture filtrate. Tyrosine appeared to be relatively effective at counteracting the effects of GAF at low concentrations,

Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Duration of treatment</th>
<th>GAF activity (Milliequivalents/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (Control)</td>
<td>0 (Initial activity)</td>
<td>9.6</td>
</tr>
<tr>
<td>Sterile storage (4 °C)</td>
<td>4 Months</td>
<td>6.5</td>
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<tr>
<td></td>
<td>10 Months</td>
<td>0.8</td>
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<tr>
<td></td>
<td>16 Months</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>32 Months</td>
<td>0.1</td>
</tr>
<tr>
<td>Autoclaving</td>
<td>30 Minutes</td>
<td>0.1</td>
</tr>
<tr>
<td>pH 2</td>
<td>Transient</td>
<td>9.6</td>
</tr>
<tr>
<td></td>
<td>7-Days</td>
<td>0.6</td>
</tr>
</tbody>
</table>

Fig. 2. TLC analysis of culture filtrates from Pseudomonas fluorescens isolates and strains. Extracts (90% ethanol) of the solids recovered from Pseudomonas culture filtrates were prepared and chromatographed on cellulose TLC plates as described in Section 2. The developed chromatograms were sprayed with ninhydrin reagent as described in the same source. The position of the Germination-Arrest Factor (GAF) band is indicated for WH6. (A) TLC analysis (on cellulose plates) of extracts from WH6 and other GAF-producing bacterial isolates. (B) TLC analysis (on cellulose plates) of extracts from WH6 and non-GAF-producing bacterial strains. (C) TLC analysis (on GHL silica plates) of extracts from WH6 and other GAF-producing bacterial isolates. (D) TLC analysis (on GHL silica plates) of extracts from WH6 and non-GAF-producing bacterial strains.
but its poor solubility limited the concentrations at which it could be tested. At higher concentrations, all of these amino acids, with the exception of glutamic acid, were themselves somewhat inhibitory to germination when tested in the absence of GAF. Interestingly, in these cases, the inhibitory effects of the amino acid itself appeared to be somewhat ameliorated in the presence of GAF, as shown in Fig. 6 for leucine.

A number of other amino acids (threonine, histidine, valine, tryptophan, asparagine, aspartic acid, and proline) exhibited somewhat weaker interactions with GAF in the *Poa* bioassay as shown in Table 3. Among the amino acids in this group, only tryptophan was able to give a germination score above 2.5 in the presence of 0.1 \(^{\text{c}}C\) culture filtrate.

Four amino acids (glycine, cysteine, lysine, and arginine) were inhibitory to germination in their own right and exhibited slight or no effect on the activity of GAF in the *Poa* bioassay. Arginine was particularly inhibitory to the germination of ABG seeds, as was lysine, the other basic amino acid.

### 4. Discussion

The GAF activity present in *P. fluorescens* WH6 culture filtrates has been shown in the present study to chromatograph with a particular ninhydrin-reactive compound that is visible on cellulose TLC plates as a purple–blue band when chromatograms of culture filtrates of *P. fluorescens* WH6 were prepared and chromatographed on cellulose and on GHL silica gel TLC plates as described in Section 2. The developed chromatograms were sprayed with ninhydrin reagent as described in the same source. The ninhydrin-stained cellulose plates are shown on the left, and the silica gel plates are shown on the right.
filtrate extracts are sprayed with ninhydrin. This ninhydrin-reactive band was present in TLC chromatograms prepared from four other GAF-producing *P. fluorescens* isolates, and it was absent in TLC chromatograms prepared from culture filtrates of four strains of *P. fluorescens* that lack the ability to produce GAF. GAF activity could not be recovered from silica gel TLC plates following fractionation of WH6 culture filtrates, because extracts of the silica gel itself were toxic in the GAF bioassay. However, on silica gel chromatograms, a broad salmon/rose-colored band that was closely associated with a narrow blue band running at a slightly higher RF could be seen with ninhydrin-staining of TLC chromatograms prepared from culture filtrates of WH6 and the four other GAF-producing *P. fluorescens* isolates. These bands were absent in chromatograms prepared from the four *P. fluorescens* strains that lack the ability to produce GAF. Thus, in addition to the demonstration that GAF activity migrates with the ninhydrin-reactive band visible on cellulose TLC plates, the presence or absence of GAF activity correlated precisely with the presence or absence of the specific ninhydrin-reactive bands described above in comparisons of GAF-producing and non-producing strains of *P. fluorescens*.

Further evidence of the identity of GAF and the ninhydrin-reactive compound visible on TLC chromatograms prepared from WH6 and other GAF-producing strains of *P. fluorescens* was obtained in studies of GAF degradation. Both GAF activity and the corresponding ninhydrin-reactive bands disappeared following autoclaving of WH6 culture filtrates. A pronounced decrease in GAF activity that occurred with prolonged storage of WH6 culture filtrates was associated with a shift in color of the corresponding ninhydrin band visible on cellulose plates (from purple-blue to green-gray). On silica gel plates, aging of WH6 culture filtrates was associated with a decrease in the salmon–rose band and a corresponding increase in the bright-blue ninhydrin band running at a slightly higher RF. Similar color changes were associated with a loss of biological activity associated with exposure of GAF to acid pH (pH 2) for several days at room temperature. Thus, the narrow blue band visible on ninhydrin-stained silica gel TLC chromatograms and the green–gray band visible on ninhydrin-stained cellulose TLC chromatograms of aged culture filtrates appear to represent a degradation product derived from GAF. This product must still retain the ninhydrin-reactive group of GAF, but based on its chromatographic behavior on silica gel, it must be slightly more hydrophobic (less hydrophilic) than GAF itself. This conclusion is based on the fact that the product moves further than GAF in the relatively hydrophilic solvent mobile phase, while GAF exhibits a greater preference for the hydrophobic silica gel matrix and moves more slowly in the mobile phase.

### Table 3

Interactions of GAF and amino acids in the standard GAF bioassay. A germination score of 1.0 indicates high GAF activity (germination completely arrested immediately after emergence of the coleorhiza and plumule). A germination score of 4.0 indicates no GAF activity (germination and seedling development equivalent to that of controls). See Section 2 for details of the bioassay procedure and the scoring system.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>WH6 culture filtrate concentration (1 × = No dilution)</th>
<th>Germination score (± Standard error of the mean) at indicated amino acid concentration (mM)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>0.0</td>
<td>0.3</td>
</tr>
<tr>
<td>Alanine</td>
<td>0</td>
<td>4.0 ± 0.0</td>
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<td>Arginine</td>
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<tr>
<td>Aspartic acid</td>
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<td>Cysteine</td>
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</tr>
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<td>Glutamic acid</td>
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<td>Glutamine</td>
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<td>Leucine</td>
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</tr>
<tr>
<td>Lysine</td>
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<td>Methionine</td>
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<tr>
<td>Valine</td>
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ninhydrin-reactive compound visible on TLC plates. Transposon mutagenesis of WH6 produced two mutations of WH6 that resulted in the loss of GAF activity in the WH6 culture filtrates, and in both cases, the loss of GAF activity was associated with a loss of the corresponding ninhydrin-reactive band from the culture filtrates of the mutant lines of WH6. Molecular genetic characterizations of the mutations in WH6-2 and WH6-3 are underway and will be reported in a subsequent publication. Preliminary results

Fig. 5. Interaction of alanine with varying concentrations of WH6 culture filtrate in the standard GAF bioassay. A germination score of 1.0 indicates high GAF activity (germination completely arrested immediately after emergence of the coleorhiza and plumule). A germination score of 4.0 indicates no GAF activity (germination and seedling development equivalent to that of controls). See Section 2 for details of the bioassay procedure and the scoring system.

Fig. 6. Interaction of leucine with varying concentrations of WH6 culture filtrate in the standard GAF bioassay. A germination score of 1.0 indicates high GAF activity (germination completely arrested immediately after emergence of the coleorhiza and plumule). A germination score of 4.0 indicates no GAF activity (germination and seedling development equivalent to that of controls). See Section 2 for details of the bioassay procedure and the scoring system.
indicate that these mutations have occurred at two different sites in the WH6 genome (Mills, unpublished), and they should provide a number of opportunities for examining the regulation and biosynthesis of GAF.

The ninhydrin-reactivity of the compound responsible for GAF activity is most easily interpreted by assuming that GAF contains an amino group. This interpretation, together with our previous demonstration (Banowetz et al., 2009) that GAF activity is associated with a low molecular weight compound that contains an acid group, suggests that GAF may be a small peptide or an amino acid analog. Based on the latter hypothesis, we have investigated the interactions of GAF with the various protein amino acids to determine if the effects of GAF might be ameliorated by one or more of these compounds. The action of GAF in arresting the germination of ABG seeds was found to be counteracted by alanine and glutamine and, somewhat less effectively, by several other amino acids. If GAF interferes with normal amino acid functions, the central role of glutamine in nitrogen assimilation in plant systems provides a ready explanation for why this amino acid would be effective in counteracting the effects of GAF. In the case of alanine, which was the most effective of all the amino acids tested in counteracting the effects of GAF, this amino acid is known to accumulate to relatively high levels in plant tissues exposed to hypoxic conditions (Good and Crosby, 1989; de Sousa and Sodek, 2003), such as might be expected to prevail in the fluid filled wells used in our bioassay system or during germination in water-soaked grass seed production fields. Presumably, this amino acid plays a particularly important role in nitrogen-shuttling under hypoxic conditions via its production by transamination of pyruvic acid produced in glycolysis. The ability of these amino acids to counteract GAF inhibition of seed germination is certainly consistent with the hypothesis that GAF may be affecting some aspect of nitrogen metabolism.

Pseudomonads are known to produce a broad spectrum of secondary metabolites, a number of which have been shown to have inhibitory effects on higher plant growth or to have antimicrobial properties (Lesinger and Margraff, 1979; Dowling and O’Gara, 1994). However, to our knowledge, none of the Pseudomonas isolates described to date exert the precise spectrum of biological effects exhibited by the GAF-producing P. fluorescens isolates that we have characterized. The failure of GAF activity to partition into ethyl acetate or other organic solvents immiscible with water (Banowetz et al., 2009) serves to distinguish it from a number of secondary metabolites, such as the phenazines or the cyclic lipopeptides, isolated from other pseudomonads (Mavrodi et al., 2006; Nielsen et al., 2002; Pedras et al., 2003; Raaijmakers et al., 2006). GAF activity is also distinct from that of the water-soluble plant-inhibiting activity reported by Kennedy and co-workers to be produced by P. fluorescens Isolate D7 (Gurusiddaiah et al., 1994). As noted previously (Banowetz et al., 2009), the ability of GAF to bind to anion exchange columns serves to distinguish it from the D7 activity. Elliott and co-workers have earlier reported the production of an unstable hydrophilic toxin by a rhizobacterial Pseudomonas isolate (RC-1) that inhibited E. coli and wheat root growth (Fredrickson and Elliott, 1985; Bolton et al., 1988). In these assays, the inhibitory activity of the toxin could be counteracted by treatment with L-methionine (Fredrickson and Elliott, 1985). The effects of this toxin on seed germination were not examined, so a direct comparison with the biological properties of GAF is not possible, but this compound appears to be somewhat more hydrophobic than GAF, based on its apparent lack of solubility in methanol and the RF values obtained with various solvent systems in tests with cellulose TLC plates (Bolton et al., 1989). Given the ninhydrin-reactivity of GAF, the recent report by Braun et al. (2008) of the isolation and purification of 3-methylarginine from culture filtrates of P. syringae pv. syringae 22d/93 is of interest. This compound appears to be responsible for the ability of P. syringae pv. syringae 22d/93 to selectively inhibit the plant pathogen P. syringae pv. glycinea. However, as shown here, arginine did not reverse the herbicidal activity of GAF, and we have not observed any effect of culture filtrates from P. fluorescens WH6 on three strains of P. syringae pv. glycinea we have tested (Halgren, unpublished). Therefore, it appears unlikely that GAF is identical to this particular amino acid analog.

The novel biological properties we have demonstrated for GAF suggest that this compound may represent a new and potentially valuable type of herbicide. The very polar character of GAF renders its purification a significant challenge, but the availability of the quantitative bioassay for GAF activity developed in our earlier work, combined with our current demonstration that this activity is associated with a particular ninhydrin-reactive compound, provides encouragement that we will ultimately be able to purify and chemically characterize the active compound.

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References


