Accumulation of *Pseudomonas*-derived 2,4-diacyethylphloroglucinol on wheat seedling roots is influenced by host cultivar

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Received 6 January 2008; accepted 17 March 2008
Available online 29 March 2008

Abstract

Production of antifungal metabolites, including the polyketide 2,4-diacyethylphloroglucinol (2,4-DAPG), is one mechanism by which biocontrol strains of *Pseudomonas fluorescens* suppress soilborne fungal pathogens. *P. fluorescens* strains vary in ability to produce 2,4-DAPG, but the role of the host in modulating metabolite accumulation in the rhizosphere is not well defined. To examine 2,4-DAPG production and accumulation during early stages of rhizoplane interactions, we compared metabolite production by two *P. fluorescens* strains in culture and on seedling roots of three *Triticum aestivum* L. (wheat) cultivars, Buchanan, Finley, and Tara, in a soil-free system. *P. fluorescens* strain Q8r1-96, an aggressive colonizer of the wheat rhizosphere, produced 1850 μg mL⁻¹ 2,4-DAPG after 48 h of growth in King’s Medium B, significantly (P > 0.05) more than 19.4 μg mL⁻¹ metabolite produced by the moderately aggressive strain Q2-87V1 under the same conditions. Rhizoplane levels of 2,4-DAPG after 4 d of Q8r1-96 colonization were 1946, 1650, and 2767 ng g⁻¹ for Buchanan, Finley, and Tara, respectively. Metabolite levels obtained for Q2-87V1 colonization were 1468, 366, and 80 ng g⁻¹ on the respective cultivars. Strain Q8r1-96 deposited significantly (P < 0.05) more 2,4-DAPG than Q2-87V1 on Tara and Finley roots, whereas both strains produced similar (P > 0.05) amounts of the metabolite on Buchanan roots. In greenhouse experiments, take-all damage was reduced only on Tara roots inoculated with Q8r1-96. To our knowledge, this is the first report to compare 2,4-DAPG accumulation in the rhizoplanes of different cultivars, and to demonstrate that rhizoplane 2,4-DAPG accumulation depends on a cultivar–bacterial strain interaction.

Published by Elsevier Inc.

Keywords: Biological control; Disease suppression; *Gaeumannomyces graminis*; PGPR; Rhizobacteria; Root colonization

1. Introduction

Strains of fluorescent *Pseudomonas* spp. suppress soilborne pathogens by several mechanisms (Bloemberg and Lugtenberg, 2001; Persello-Cartieaux et al., 2003), including the production of antifungal metabolites in the rhizosphere (Fakhouri et al., 2001; Haas and Keel, 2003; Weller et al., 2007). In the Pacific Northwest of the United States, certain strains of *Pseudomonas fluorescens* Migula 1895 suppress *Gaeumannomyces graminis* (Sacc.) Arx & D. Olivier var. *tritici* Walker (Ggt), the causal agent of take-all of wheat, and coincide with the phenomenon of take-all decline (Weller et al., 2002). Ggt attacks seedling roots, causing loss of seminal and tiller roots, root lesions, stunting of shoots, chlorosis, and eventual collapse of the vascular system that results in death of the plant (Cook, 2003). Suppressive strains of *P. fluorescens* produce the polyketide antibiotic 2,4-diacyethylphloroglucinol (2,4-DAPG). 2,4-DAPG is one of most extensively-studied biocontrol metabolites synthesized by fluorescent pseudomonads (Bangera and Thomashow, 1999; Delany et al., 2001; Keel et al., 1999). However, the mode of action of 2,4-DAPG is not known. Its broadly antifungal (Keel et al., 1992) and
antibacterial activities (Tada et al., 1990), as well as its phytotoxicity (Keel et al., 1992; Fujimoto et al., 1995) suggest that 2,4-DAPG targets a universal structural component or process of the cell, such as cytoplasmic structure (De Souza et al., 2003).

Production and accumulation of 2,4-DAPG is governed by Pseudomonas genes that regulate motility, colony phase variation, and other traits critical to intra-population signaling and colony maturation (Lugtenberg et al., 2001; Péchy-Tarr et al., 2005; Schnider et al., 1995), as well as by complex rhizosphere interactions (Mavrodi et al., 2004; Notz et al., 2002; Schnider-Keel et al., 2000), mineral composition of the environment (Duffy and Défago, 1997), soil type (Bonsall et al., 1997), and whether metabolite accumulation was cultivar-dependent. The term rhizosphere is used to distinguish roots in a soil-free system. In contrast to previous studies, we have examined metabolite deposition during early stages of root colonization and under conditions that limit the impact of soil-based biotic and abiotic factors. We also have developed a means of quantifying take-all damage in 14-day-old wheat seedlings. Here, we report that the amount of rhizoplane 2,4-DAPG produced by Q8r1-96 was greater than that produced by Q2-87V1, but the deposition of the metabolite by each strain varied according to host genotype. Quantitation of 2,4-DAPG in a soil-free root system will provide the basis for host cultivar selection and amounts of metabolite to apply to roots in gene expression profiling experiments.

2. Materials and methods

2.1. Bacterial strains and cultures

Pseudomonas fluorescens Q8r1-96 and Q2-87 were isolated from a take-all-suppressive soil from Quincy, WA (Raaijmakers et al., 1999). Q2-87V1 was isolated as a natural variant of Q2-87 that showed reduced 2,4-DAPG production in culture. This variant was identical to Q2-87V1 (Raaijmakers et al., 1999) were stored as 1/3 KMB broth in an autoclaved 50-mL Erlenmeyer flask was diluted to an A600 of 0.1 (about 10⁸ CFU mL⁻¹) with KMB. Twenty-five milliliters of fresh Q8r1-96 and Q2-87V1 starter cultures were grown at 23 ± 1 °C for use in experiments.

For 2,4-DAPG measurements from cultured bacteria, Q8r1-96 and Q2-87V1 starter cultures were grown at 28 °C for 16–18 h in 4 mL of full-strength, non-supplemented KMB broth, and diluted to an A₆₅₀ of 0.1 (about 10⁹ CFU mL⁻¹) with KMB. Twenty-five milliliters of fresh KMB broth in an autoclaved 50-mL Erlenmeyer flask was inoculated with 1 mL of diluted cells. Cultures were grown at 28 °C at 250 rpm, and sampled in triplicate at 4-h intervals from 0 to 48 h as follows: a 3-mL aliquot was removed...
from each culture, rapidly chilled on ice, and stored at −20 °C for optical density (growth) readings. A_600 was monitored for 100 µL of undiluted and diluted (1:10) samples using a microplate spectrophotometer (Dynatech MR5000, Dynatech Laboratories, Inc., Alexandria, VA). The experiment was done once.

2.2. Seed inoculations and growth of seedlings

_Pseudomonas fluorescens_ Q8r1-96 and Q2-87V1 were scraped from agar plates grown for 4 days at 23 ± 1 °C. Bacteria were suspended in 1 mL of sterile nanopure water, washed twice in the same, adjusted to an A_600 of 0.10 (about 10^7 CFU mL^-1), and used without dilution or as 1:10, 1:100, and 1:1000 aqueous dilutions (McSpadden Gardener et al., 2001; Okubara et al., 2004).

Seed of _Triticum aestivum_ cultivars Buchanan, Finley, and Tara were obtained from Washington State Crop Improvement Association, Pullman, WA. Seeds were not surface-sterilized prior to the experiments. Batches of 100 seeds were inoculated with 10^4, 10^5, 10^6, and 10^7 CFU of each bacterial strain (10 µL of diluted or non-diluted bacteria in 0.5 mL 1% methyl cellulose), for final inocula densities of 10^2, 10^3, 10^4, and 10^5 CFU seed^-1. Control treatments consisted of 0.5 mL 1% methyl cellulose. Seeds were tumbled in autoclaved flasks until they did not adhere to each other or to the flask, and dried in a laminar flow hood for 2–3 h prior to germination (Okubara et al., 2004).

For metabolite quantitation and rhizoplane population density measurements, 250, 300, and 150 treated seeds of Buchanan, Finley, and Tara, respectively, were germinated in sterile 150 mm disposable Petri plates lined with two layers of Whatman No. 1 filter disks, at a density of 18–20 seeds per plate. The disks were moistened with 8 mL of autoclaved water before addition of seeds. Seedlings were maintained in darkness for 4 days at 23 ± 1 °C prior to bacterial population density measurements and 2,4-DAPG quantitation. Each treatment consisted of a combination of bacterial strain, bacterial inoculum density and cultivar. The treatments were repeated five times for Q8r1-96 and three times for Q2-87V1.

2.3. Bacterial population density measurements

Rhizoplane populations of _P. fluorescens_ and total culturable heterotrophic bacteria were quantified for each inoculum level (10^2–10^5 CFU seed^-1) in four of the five experiments using Q8r1-96 and in all three experiments using Q2-87V1 (described in Section 2.2). Three batches of six roots each were harvested at 4 days for each inoculation (triplicate samples). Excised roots were pooled and suspended in 10 mL of sterile distilled water for quantitation by the dilution end-point method (Landa et al., 2002a). Bacteria were recovered by 1 min of agitation on a vortex mixer, followed by 1 min of sonication. Sixteen serial 1:2 dilutions, starting with 100 µL of aqueous root extract in 200 µL of sterile distilled water, were performed in 96-well microtiter plates. Fifty microliters of each dilution was transferred to 200 µL of either 1:10 tryptic soy broth (TSB) supplemented with 100 µg mL^-1 cycloheximide (for total heterotrophic bacteria), or 1/3 KMB^rif^ broths (for Q8r1-96 and Q2-87V1). A_600 was monitored after 48 h for TSB cultures and 72–74 h for KMB cultures using a Dynatech MR5000 microplate spectrophotometer (Dynatech Laboratories, Inc.). The most dilute (endpoint) sample in the dilution series that gave readings of >0.012 absorbance units above background was used to calculate population density (Okubara et al., 2004). Rhizoplane population densities were the average of triplicate measurements, expressed as CFU of RifR bacteria normalized to gram fresh weight of root tissue.

Rif^R_ bacteria recovered from root tissues were analyzed using restriction fragment length polymorphisms of the _phiD_ gene. _phiD_ segments were amplified from the bacteria in two randomly-selected endpoint samples per treatment, and subjected to _Hae_III restriction fragment analysis (McSpadden Gardener et al., 2001).

2.4. Root growth quantitation

To quantitate root growth in Buchanan, Finley, and Tara seedlings, seeds of each cultivar were coated as described in Section 2.2 with 10^5 CFU seed^-1 of either _P. fluorescens_ Q8r1-96 or Q2-87V1 in 1% methyl cellulose, or 1% methyl cellulose alone. Three 150-mm diameter Petri plates containing 18–20 seeds plate^-1 were grown at 23 ± 1 °C for 4 days. Digital scans of 20 roots randomly selected from the plates were obtained for each cultivar and seed treatment using a HP ScanJet 5370C (Hewlett Packard, Palo Alto, CA) at a resolution of 300 pixels sq. in. Morphometric variables of total root length (cm), mean root diameter (mm), surface area (cm^2), and root volume (cm^3) were quantified at no more than 1× magnification using the pixel enumeration software Win-RHIZO 6.0 (Regent Instruments, Inc., Quebec, Canada).

2.5. 2,4-DAPG extractions and HPLC photodiode array (PDA) spectroscopy

Each 2,4-DAPG extraction was done using 4.0 g fresh weight of root material collected from 190, 240, and 90 treated seedlings of Buchanan, Finley, and Tara, respectively. Roots were excised within a few millimeters of the seed using sterilized scissors, and directly collected in a 150 mL glass beaker on ice. Harvested roots were transferred to autoclaved Erlenmeyer flasks and stored at −20 °C prior to 2,4-DAPG extraction.

Extractions were carried out as described previously (Bonsall et al., 1997; Raaijmakers et al., 1999), except that the number and duration of extractions were reduced. Roots were suspended in 10 mL of 80% acetone that was acidified to pH 2.0 with 113 µL of 10% trifluoroacetic acid (TFA). All samples except blanks received 2 µg phenazine

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The HPLC PDA system consisted of a 717 Plus auto sampler, 600 controller, C18 reverse phase HPLC (4 μm symmetry column, 8 x 100 mm for bacterial cultures; 5 μm symmetry column, 3.5 x 150 mm for roots), a 600E solvent delivery system, and a 996 PDA detector (Waters Corporation, Milford, MA). HPLC was carried out as described by Bonsall et al. (1997), with the following modifications. 2,4-DAPG extracts were suspended in 1 mL of 35% acetonitrile (ACN), 0.1% TFA. Each sample was centrifuged for 10 min at 12,000 g to remove particulates. For bacterial cultures, 55 μL injections were used for each sample. 2,4-DAPG was partitioned using a 2-min initialization of 10% TFA, 0.1% ACN followed by a 28-min linear gradient to 99.9% ACN, 0.1% TFA, at a flow rate of 1.0 mL min\(^{-1}\) (gradient curve profile #6, Waters Corp.). For root extracts, 30 μL injections were used for each sample, and metabolite was partitioned as described for bacterial cultures, except using a flow rate of 0.5 mL min\(^{-1}\) (gradient curve profile #5, Waters Corp.). HPLC gradient profiles were monitored at 270 nm \(\lambda_{max}\) for 2,4-DAPG, and 247 nm \(\lambda_{max}\) for the phenazine internal standard. The HPLC PDA detection limit was 5 ng of 2,4-DAPG.

A 2,4-DAPG standard curve was generated by spiking 4 g untreated Tara root tissue with 0.2, 0.5, 1.0, 2.0, 4.0, 6.0, 8.0, and 5.0 μg of metabolite. Two micrograms of phenazine (internal standard) was added to each sample. Each point on the standard curve was the mean of eight experiments; an experiment consisted of one sample of each concentration. A negative control without added 2,4-DAPG was included in each experiment. Examinations were performed as described in the previous paragraph. Extraction efficiencies were consistent for all samples and experiments, and averaged 61%. Variance (scedasticity) among the highest (10 μg) or lowest (200 ng) data points was determined using the F-test and analysis of variance (Statistix vers. 8.1, Tallahassee, FL). The sum of relative errors, and fit of the curve through the origin within a 99% confidence interval indicated that the best weighting factor was 1/x\(^{0.5}\) (Kiser and Dolan, 2004; XLSTAT-Pro vers. 7.1). Therefore, all 2,4-DAPG data points were expressed as a product of this factor. The relationship between peak area (y) and input (spiked) 2,4-DAPG (x) was linear, and described by the equation \(y = 326.787x + 32.046\), where \(R^2 = 0.9987\).

### 2.6. Quadrupole time-of-flight mass spectroscopy

The HPLC/PDA/Quadrupole time-of-flight (Q-Tof) mass spectrometer system consisted of a Waters 2695 Alliance HPLC, 996 PDA detector, and Q-Tof II mass spectrometer (Waters Corporation, Milford, MA). Extractions were carried out as above, except that each sample was brought up to 500 μL of 98% ACN, 2% acetic acid. Thirty microliters injections were used on a C18 reverse phase symmetry column (5 μm, 3.5 x 150 mm, Waters Corporation, Milford, MA). HPLC solvent conditions were a flow rate of 400 μL min\(^{-1}\) with a 2 min initialization at 10% ACN, 2% acetic acid followed by a 20 min linear gradient to 98% ACN, 2% acetic acid. HPLC Q-Tof conditions consisted of a corona current of 2.7 μA, cone voltage of 25 V, collision energy of 12, source temperature of 120 °C, and probe temperature of 650 °C. HPLC PDA gradient profiles were monitored at 270 nm and Q-Tof profiles were monitored at 211.0606 exact mass (Tof) positive ion mode atmospheric chemical ionization (APCI). The detection limit of the Q-Tof system was 15 ng of 2,4-DAPG.

### 2.7. Ggt suppression assays

*Gaeumannomyces graminis* var. *tritici* (Ggt) isolate R3-11la-1 (Pierson and Weller, 1994) was cultured on potato dextrose agar. Agar blocks (~1 cm\(^2\)) were added to twice-autoclaved whole oats in 1-L flasks and incubated at room temperature for 4–5 weeks (Raaijmakers and Weller, 2001). Oats colonized with Ggt were air-dried, milled to homogeneity in a coffee grinder and sieved prior to use as inocula.

Ggt inoculum (1% w/w) was combined with sieved Shano sandy loam obtained from a non-cropped site near Quincy, WA, and mixed by thorough shaking in a large plastic bag. The physical, chemical and microbial composition of this soil has been described by Raaijmakers et al. (1997). Non-infested soil served as a control. About 50 mL of soil was transferred to 1-in. x 6-in. cones (Stuewe & Sons, Corvallis, OR). Immediately before sowing, the soil in each cone was drenched with 50 mL of an aqueous solution of metalaxyl (75 mg L a.i.\(^{-1}\)) to suppress *Pythium* and other Oomycetes, and allowed to drain completely. To facilitate root harvest, a longitudinal slit was made in each cone and the slit secured with tape before planting.

Each cone containing non-infested or Ggt-infested soils was sown with one seed of Buchanan, Finley or Tara coated with either *P. fluorescens* Q8r1-96 or Q2-87V1 at an inoculum level of 10\(^5\) CFU seed\(^{-1}\), or 1% methyl cellulose (control). Seventeen to 21 plants of each cultivar were sown for each of the following seed and soil treatment combinations: (1) *P. fluorescens*—non-infested soil; (2) methyl cellulose—Ggt-infested soil; (3) *P. fluorescens*—Ggt-infested soil; (4) methyl cellulose—non-infested soil. Eight to twelve plants were harvested for disease evaluations, and nine plants (three pools of three plants per pool) were
sampled for bacterial population density measurements as described in Section 2.3.

Seeds were covered with 5 mL per cone of non-infested soil. Plants were maintained at 15 ± 1°C, with 12 h daily supplemental lighting (66–90 μmol m–2 s–1). Cones were watered on alternate days or as needed using 10 to 20 mL of water; every third watering was done using 0.94 g L–1 Scott’s Miracle-Gro (Marysville, OH) fertilizer solution. Watering was withheld 3 days prior to harvest. After 14 days, soil and roots were removed from the cones and gently rinsed with water until the soil was completely removed. The length of the first true leaf and fresh root weight were determined for each plant. Digital images of roots were analyzed as described in Section 2.4. Values for bacterial and pathogen treatments are expressed relative to methyl cellulose controls (1.00). The experiment was repeated twice.

2.8. Statistical analyses

Comparisons of bacterial population densities (log CFU) or 2,4-DAPG measurements normalized to root weight, CFU of bacteria, root surface area or per seedling were conducted for the cultivars and _P. fluorescens_ strains using analysis of variance, and mean comparisons among samples were performed using Fisher’s protected least significant difference (LSD) test at _P_ < 0.05 (Statistix). Interactions between experiment, cultivar and bacterial strain were analyzed using the general analysis of variance function (Statistix) and either log CFU or normalized 2,4-DAPG measurements as dependent variables. Bartlett’s _t_-test for equal variances was applied to determine whether the same treatments from separate experiments could be combined. Seedling root morphometric data and _Ggt_ disease severity variables (shoot and root lengths, root weight and average root diameter) also were compared using Fisher’s protected least significant difference (LSD) test at _P_ < 0.05 (Statistix vers. 8.1).

3. Results

3.1. Differential production of 2,4-DAPG by _Q8r1-96_ and _Q2-87V1_ in culture

_Pseudomonas fluorescens_ strains _Q8r1-96_ and _Q2-87V1_ displayed markedly different profiles of 2,4-DAPG production when cultured in KMB (Fig. 1A). Strain _Q8r1-96_ produced detectable amounts of the metabolite at 4 h and maximal amounts of 1850 μg mL–1 between 20 and 24 h. In contrast, _Q2-87V1_ produced detectable metabolite only after 12 h and the metabolite continually accumulated during the 48-h sampling period. 2,4-DAPG production by _Q2-87V1_ was 19.4 μg mL–1 at 48 h, yet the growth of _Q2-87V1_ exceeded that of _Q8r1-96_ at that time (Fig. 1B). These findings indicate that the strains differ in the dynamics of 2,4-DAPG production and growth rate in KMB.

![Fig. 1. Production of 2,4-DAPG by _P. fluorescens_ Q8r1-96 (black) and Q2-87V1 (white) in KMB medium.](image)

In all but one case, rhizoplane population densities of both _Q8r1-96_ and _Q2-87V1_ exceeded 10⁸ cells g⁻¹ on all cultivars at 4 days post-inoculation (dpi) (Table 1). Overall, no significant inoculum-by-experiment (_P_ = 0.212–0.916) or inoculum-by-cultivar (_P_ = 0.299–0.933) interactions were observed for population densities of either bacterial strain. The exception was _Q2-87V1_ at 10⁵ CFU seed⁻¹ on Tara (Table 1) which averaged log 7.61 (4.1 ± 10⁵ cells g⁻¹), significantly (_P_ = 0.002) lower than population densities obtained at other inocula levels. Population densities of total culturable heterotrophic bacteria ranged from log 7.55 to log 9.46 for all cultivars and bacterial inoculum levels. _RifR_ bacteria recovered from seedling roots exhibited the _phiD-HaeIII_ restriction fragment length polymorphisms (McSpadden Gardener et al., 2001) typical for _Q8r1-96_ or _Q2-87_, as inoculated. Non-inoculated roots yielded no _RifR_ bacteria in endpoint dilution plate assays, and cultured material did not yield PCR products. These
assays indicated that the RifR bacteria originated from the seed inoculum, and that the introduced strains were predominant in colonized roots. Extracts of non-inoculated roots did not yield phiID PCR products (data not shown).

3.3. Rhizoplane 2,4-DAPG after colonization by strains Q8r1-96 and Q2-87V1

2,4-DAPG was quantified in samplings of the same roots used to determine bacterial population densities. The metabolite was quantifiable on seedling roots of Buchanan, Finley, and Tara at all Q8r1-96 inoculum levels, but only at the highest level (10⁵ CFU seed⁻¹) of Q2-87V1. *P. fluorescens* Q8r1-96 yielded higher rhizoplane amounts of 2,4-DAPG compared to Q2-87V1 on all cultivars (Fig. 2). Metabolite measurements from separate experiments were combined because variances among experiments were equal in Bartlett’s t-tests, with no significant (P > 0.05) treatment-by-experiment interactions. 2,4-DAPG levels were 1946 ± 250, 1650 ± 210, and 2767 ± 523 ng g⁻¹ for Q8r196-treated Buchanan, Finley, and Tara, respectively, compared to 1468 ± 111, 366 ± 108, and 80 ± 45 ng g⁻¹ for Q2-87V1 treatments (Fig. 2A). Metabolite levels normalized on the basis of 10² CFU were 9.1 ± 2.3, 10.1 ± 2.1, and 22.8 ± 2.0 ng (10⁷ CFU)⁻¹ for Q8r1-96 on Buchanan, Finley and Tara, respectively, and 9.9 ± 0.6, 1.0 ± 0.4, and 1.0 ± 0.4 for Q2-87V1 on the same cultivars (Fig. 2B).

3.4. Rhizoplane 2,4-DAPG accumulation and wheat cultivar

Significant cultivar-by-strain interactions for DAPG measurements normalized on the bases of root weight, 10⁷ CFU bacteria, seedling number and root surface area (P = 0.0066; 0.0014; 0.0001; 0.0027, respectively) were detected. Roots of Tara colonized by Q8r1-96 supported about 35 times more metabolite on a per-gram basis (Fig. 2A) and per-10⁷ CFU basis (Fig. 2B) than those colonized by Q2-87V1, a difference that reflected relative production levels in culture. In contrast, Buchanan roots displayed similar amounts of the metabolite upon colonization by either strain, indicating that inherent ability to produce the metabolite was modulated by cultivar.

2,4-DAPG accumulation was calculated on a per-seedling basis. A clear distinction between Tara and the other two cultivars remained; the average metabolite level of 123 ± 23 ng seedling⁻¹ for Tara was significantly (P < 0.05) higher than those of 41 ± 5 and 28 ± 3 ng seedling⁻¹ for Buchanan and Finley, respectively. Also, levels on Q2-87V1-treated roots (31 ± 2, 6.1 ± 1.8, and 3.6 ± 2.0 ng seedling⁻¹ for the respective cultivars) were consistently lower than those for Q8r1-96.

Root length, surface area and other morphometric variables did not differ among Q8r1-96, Q2-87V1 or methyl cellulose control treatments for any of the cultivars (Table 2). Tara roots were more robust than those of Buchanan and Finley, a characteristic that was reflected in its root surface area (Table 2). Total root surface area, calculated by multiplying the average surface area per root by the number of roots used for metabolite extraction, was used to normalize 2,4-DAPG. Despite normalization to total root surface area, Tara still exhibited the highest amount of accumulated metabolite for Q8r1-96, 34.3 ± 6.5 ng cm⁻² compared to 23.2 ± 3.0 and 14.4 ± 1.8 ng cm⁻² for Buchanan and Finley, respectively, and the least amount for Q2-87V1, 1.0 ± 0.6 ng cm⁻² compared to 17.5 ± 1.3 and 3.7 ± 0.9 ng cm⁻² (Buchanan and Finley, respectively).

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Inoculum (CFU seed⁻¹)</th>
<th>Log population densitya</th>
<th>Q8r1-96</th>
<th>Q2-87V1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buchanan</td>
<td>10⁵</td>
<td>9.10 ± 0.48</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10⁴</td>
<td>8.99 ± 0.37</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10³</td>
<td>9.25 ± 0.52</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10²</td>
<td>9.29 ± 0.48</td>
<td>9.03 ± 0.51</td>
<td></td>
</tr>
<tr>
<td>Finley</td>
<td>10⁵</td>
<td>9.08 ± 0.54</td>
<td>8.50 ± 0.32</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10⁴</td>
<td>9.11 ± 0.40</td>
<td>9.16 ± 0.26</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10³</td>
<td>9.17 ± 0.50</td>
<td>9.42 ± 0.09</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10²</td>
<td>8.84 ± 0.53</td>
<td>9.04 ± 0.56</td>
<td></td>
</tr>
<tr>
<td>Tara</td>
<td>10⁵</td>
<td>8.70 ± 0.51</td>
<td>7.61 ± 0.44a</td>
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</tr>
<tr>
<td></td>
<td>10⁴</td>
<td>8.85 ± 0.47</td>
<td>8.59 ± 0.45</td>
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<tr>
<td></td>
<td>10³</td>
<td>9.05 ± 0.31</td>
<td>8.59 ± 0.49</td>
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<tr>
<td></td>
<td>10²</td>
<td>9.11 ± 0.41</td>
<td>8.40 ± 0.61</td>
<td></td>
</tr>
</tbody>
</table>

a Means and standard deviations of 6 seedlings, obtained from four experiments using Q8r1-96 and three experiments using Q2-87V1. For each experiment, three replicates of 6 seedlings per treatment were assayed.

b Not determined.

* Significant (P < 0.05) difference in the mean with respect to all other means in the table, based on Fisher’s protected LSD.
Table 2
Average surface areas of 4-days-old seedling roots of cultivars Buchanan, Finley, and Tara, quantified from digital images using WinRHIZO software

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Surface area (cm²)</th>
<th>2,4-DAPG (ng cm⁻²)¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Buchanan</td>
<td>Finley</td>
</tr>
<tr>
<td>Q8r1-96</td>
<td>1.76 ± 0.33</td>
<td>1.90 ± 0.52</td>
</tr>
<tr>
<td>Q2-87V1</td>
<td>1.39 ± 0.51</td>
<td>1.67 ± 0.55</td>
</tr>
<tr>
<td>1% MC</td>
<td>1.58 ± 0.50</td>
<td>1.54 ± 0.42</td>
</tr>
</tbody>
</table>

¹ Mean and standard error of 20 seedling roots per treatment.
² Total root surface area was determined after treatment with 10⁵ CFU seed⁻¹ P. fluorescens Q8r1-96 or Q2-87V1 in 1% methyl cellulose, or 1% methyl cellulose only (MC).
³ Indicate significant (P < 0.05) differences relative to Buchanan and Finley, based on Fisher’s protected LSD.

3.5. Authentication of 2,4-DAPG using Q-Tof mass spectroscopy

The relatively low levels of metabolite that were detected in Q2-87V1-colonized roots of Finley and Tara using HPLC PDA spectroscopy led us to analyze the extracts using quadrupole time-of-light mass spectroscopy (Q-Tof MS), a more specific analytical method. Q-Tof MS unambiguously detected a species of mass of 211.0606, an exact match to the 2,4-DAPG standard (data not shown). We concluded that the metabolite quantified by HPLC PDA spectroscopy was 2,4-DAPG.

2,4-DAPG was not detectable in extracts of roots treated with Q2-87V1 at 10²–10⁴ CFU seed⁻¹ using HPLC PDA spectroscopy. We sought to determine whether the metabolite was being masked by another compound having the same absorbance maxima. Q-Tof MS confirmed nil or trace levels of the metabolite, indicating that the absence of 2,4-DAPG at lower Q2-87V1 inoculum levels had a biological rather than analytical basis.

3.6. Suppression of take-all on Q8r1-96-colonized Tara roots

To determine whether differences in rhizoplane 2,4-DAPG accumulation had consequences to root growth in the presence of the take-all pathogen, Ggt, we assayed the effect of the P. fluorescens strains on take-all damage in 14-day-old, soil-grown seedling roots in the greenhouse. In past studies, take-all suppression by fluorescent pseudomonads (e.g., Ownley et al., 1992; Pierson and Weller, 1994) was evaluated using semi-quantitative visual measurements made after 3–5 weeks. We conducted two 3-week experiments to assess the effect of seed-coated bacterial treatments on Ggt, but saw no significant (P > 0.05) differences in take-all disease severity among the three cultivars (data not shown). In our 14-days studies, take-all symptoms, including chlorosis, brown root lesions and dark runner hyphae, that typically are observed at later times had not developed. However, we observed differences in foliar growth at 14 days, and quantified take-all severity on the bases of length of the first leaf and root fresh weight (Table 3), variables that have been used to monitor disease suppression in other biocontrol interactions (Keel et al., 1992; Raaijmakers and Weller, 1998).

4. Discussion

The secondary metabolite 2,4-DAPG is produced by effective biocontrol strains of P. fluorescens, including Q8r1-96, that colonize the rhizospheres of wheat and other host plants. Strain Q8r1-96 produced maximal amounts of 2,4-DAPG at 20 and 24 h in our culture conditions. This profile was similar to that obtained in an independent experiment in which metabolite was monitored at 24 and 48 h (D. Mavrodi, unpublished data), and for P. fluorescens CHAO grown in an ammonium–glucose medium (Schnider-Keel et al., 2000). Our findings indicate that strain Q8r1-96, an aggressive colonizer in a previous cultivar survey (Okubara et al., 2004), yielded more metabolite in culture and on seedling roots compared to the less aggressive Q2-87V1.

The role of the host in governing 2,4-DAPG production and other aspects of rhizobacterial interactions is largely unexplored, in part due to difficulties in working with roots in soil. We adopted a soil-free system initially to examine wheat root genes regulated by rhizobacteria, and have used...
it to compare 2,4-DAPG accumulation by two *P. fluorescens* strains on roots of three wheat cultivars. Cultivars Buchanan and Tara yielded contrasting patterns of rhizoplane metabolite accumulation. These patterns were observed whether metabolite was normalized to root weight, bacterial population density, seedling, or root surface area. Microscopic morphological characteristics such as root hair number, quantity and quality of root exudates (Schnider-Keel et al., 2000) or cultivar-specific differences in root physiology that impact 2,4-DAPG synthesis or degradation might account for the patterns. Alternatively, non-introduced microbes that are present in the spermatophore might account for the patterns. Alternatively, non-introduced microbes that are present in the spermatophore or rhizosphere of specific cultivars might differ among cultivars, and were about 1–2 log decades higher relative to Tara. The suppression of *Ggt* damage in roots of older seedlings. Quantitation of metabolite in roots from greenhouse assays would provide a comparison of rhizosphere and rhizoplane accumulation. However, the long-term consequences of high metabolite accumulation at a single time point cannot be predicted, and disease suppression as well as bacterial population density will be impacted by environmental factors at later times.

At early stages of infection, *Ggt* causes loss of seminal root mass and stunting (Cook, 2003). Suppression of take-all is correlated to production of 2,4-DAPG in the wheat rhizosphere at rates of about 1 μg g⁻¹ root (Keel et al., 1992) with a minimum of 0.25 μg g⁻¹ root, or 62 ng (10⁶ CFU⁻¹) (Raaijmakers et al., 1999). Metabolite production is likely to be most critical during early seedling development when roots are most susceptible to pathogen attack. In interactions with both Q8r1-96 and Q2-87V1, Buchanan and Finley supported less rhizoplane metabolite relative to Tara. The suppression of *Ggt* damage in roots of 14-days-old Tara seedlings suggests that the capacity of host roots to harbor high amounts of the metabolite early during the biocontrol interaction has a protective effect in older seedlings. Quantitation of metabolite in roots from greenhouse assays would provide a comparison of rhizosphere and rhizoplane accumulation. However, the long-term consequences of high metabolite accumulation at a single time point cannot be predicted, and disease suppression as well as bacterial population density will be impacted by environmental factors at later times.

Our findings add another layer of complexity to the action of antifungal metabolites in agricultural soils and open new questions about host responses to biocontrol *P. fluorescens*. For example, the impact of host cultivar on rhi-
zosphere population structure, including Ggt, and on the robustness of take-all decline can be explored. Experiments are under way to determine whether 2,4-DAPG triggers defense or stress responses (Iavicoli et al., 2003) or induced systemic resistance (Verhagen et al., 2004) in wheat, whether bacterial strains differ in ability to induce changes in wheat gene expression, and whether plants respond to applied 2,4-DAPG in a cultivar-dependent manner. Ultimately, host genes controlling metabolite accumulation will be sought with the view to modulating specific host determinants that improve the robustness of wheat–P. fluorescens interactions under natural conditions.

Acknowledgments

The authors thank James Mitchell for expertise in HPLC PDA, Mike Berghammer for colonization analysis, John Burns for the gift of wheat seeds, Richard Allerdge for advice on statistics, and David Weller and Kurtis Schroeder for advice on take-all. 2,4-DAPG was synthesized by Patrice Marchand. This work was supported by USDA ARS Project Number 5248-22000-012-00D. References to a company and/or product by the USDA are only for the purposes of information and do not imply approval or recommendation of the product to the exclusion of others that may also be suitable.

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