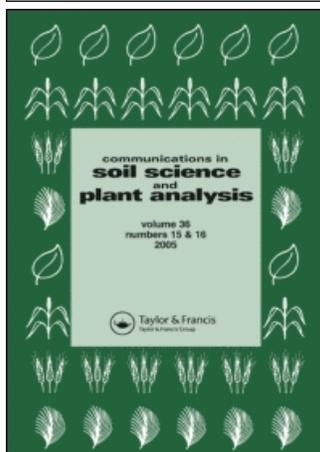


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Novel Screen for Investigating In Situ Rhizosphere Production of the Antibiotic 2,4-Diacetylphloroglucinol by Bacterial Inocula

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Abstract: The rhizosphere is a complex zone of multitrophic interactions comprising plant roots, associated bacteria, fungi, and micro-, meso-, and macro-fauna. Of considerable importance in this system is the production of antibiotics by root-associated or “rhizo” bacteria. This is a widespread phenomenon of which a much-studied exemplar is the production by pseudomonads of 2,4-diacetylphloroglucinol (DAPG), known to be effective in the suppression of soil-borne fungal pathogens. Rapid advances in understanding the molecular and biochemical bases of antibiotic (particularly DAPG) production have been made. However, our understanding of in situ antibiotic production currently lags behind this. There is therefore a need for a rapid soil-based screen with which to identify antibiotic producers under rhizosphere C-flow conditions. Here, a novel “rhizocosm,” comprising porous pipe and Rhizon sampler[®], was superior to a simple, nonperfusing incubation-type microcosm with respect to supporting a rhizobacterial inoculum. Its use as a screening tool is illustrated by screening known DAPG-producing inocula in soil continuously supplied with simulated rhizosphere carbon flow. The DAPG was then extracted from soil using acetone and quantified by high-performance liquid chromatography (HPLC). Simple sugars (glucose and fructose) stimulated the greatest DAPG production, while

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glucose with an additional organic or amino acid, or with a signal molecule, resulted in strongly variable DAPG expression.

Keywords: Antibiotic production, DAPG, *Pseudomonas fluorescens*, rhizocism

INTRODUCTION

The rhizosphere is a complex ecosystem encompassing many biological and biochemical interactions between plant roots, soil microbiota, and the soil physical environment. The majority of these interactions are driven by plant-derived carbon (C), which also plays a key role in determining rhizosphere microbial diversity and function (Standing et al. 2005). Rhizosphere carbon comprises low-molecular-weight compounds (sugars, amino acids, organic acids, simple phenolics) and high-molecular-weight compounds (e.g., complex polysaccharides and peptides), which are substrates for soil heterotrophic microbial growth. Within the rhizosphere microbial community, the fluorescent pseudomonads comprise metabolically and functionally diverse strains (Lugtenberg and Dekkers 1999), many of which promote crop plant growth and health. Campbell and Greaves (1990), for example, showed that out of 150 wheat root *Pseudomonas* isolates, 40% stimulated root growth, an equal percentage inhibited root growth, and the remainder had no effect. Certain rhizosphere *Pseudomonas* strains produce secondary metabolites that are effective antibiotics and thus have received much attention for their potential as biocontrol agents (Haas and Keel 2003; Weller et al. 2002; Whipps 2001; Thomashow and Weller 1996). This study investigates the production of 2,4-diacetylphloroglucinol (DAPG, a phenolic metabolite of the polyketide group) by various *Pseudomonas* strains in response to rhizosphere carbon (C) components (sugars, organic/amino acids, and signal molecules) introduced into natural soils via an artificial root. The DAPG is a broad-range antibiotic with antibacterial antifungal, anti-helminthic, and phytotoxic properties (Thomashow and Weller 1996) that is particularly efficacious against the root-disease fungal pathogen *Gaeumannomyces graminis* var. *tritici* (Take-all). The DAPG-producing *Pseudomonas* strains are widely distributed and have been isolated from sites in North America, Europe, and Africa (Keel et al. 1996). Duffy and Defago (1999) and Shanahan et al. (1992) showed the selective in vitro effects of different C sources and minerals on DAPG production. The effects of root exudates on the growth and physiology of the soil microbial community are, therefore, of great interest and a fuller understanding of these effects presents an opportunity to manipulate rhizosphere processes, that is, for biocontrol.

There are many bench scale containers for the study of rhizosphere processes (Wenzel et al. 2001; Griffiths et al. 1998), but one defining limitation of their construction is outer wall nonporosity. Soil water, therefore,

will be primarily under the influence of gravity, and lateral flow will not be taken into account. Depending on the questions being asked, this may result in experimental artifacts. A novel rhizocosm, comprising porous pipe and Rhizon samplers[®] (Rhizosphere Research Products, Wageningen, the Netherlands), is presented here. Its advantages lie in ease of construction, high throughput potential, and low cost. Furthermore, its relatively small volume means that a relatively homogenous plant-free rhizosphere can be created. This has significant advantages for the screening of the responses of microbial populations or communities to specific stimuli.

The utility of the novel rhizocosm is illustrated with pseudomonads and antibiotic production. We show the utility of our rhizocosm design in using perfusion to maintain an active microbial population and thus a more realistic environment than that created in standard closed batch microcosms. This system, has wider applicability for screening in situ microbial production of interesting metabolites and microbial process level function. Importantly, bacterial inocula were challenged not only with substrate C but also with signal molecules. We also show that different strains respond to different C sources in terms of soil-extractable DAPG and discuss this with respect to the soil environment.

MATERIALS AND METHODS

Rhizocosm Chamber Construction and Operation

Each rhizosphere chamber or rhizocosm was constructed by cutting a 30-cm length of porous rubber pipe (Leaky Pipe[®]) (Leaky Pipe Systems Ltd, Maidstone, U.K.), which was then washed several times in deionized water and dried at 45°C to remove any water-miscible and volatile contaminants. The rhizosphere chamber was then sealed at one end with a plastic stopper and carefully packed with 25 g fresh weight of a steam-sterilized or nonsterile agricultural loamy sand soil (pH 5.5–5.8; total C, 2.4%; total nitrogen (N), 0.25%; biomass C (mg C 100 g⁻¹), 75.6; and cation exchange capacity (CEC) (cmol kg⁻¹), 7.5 at 27% volumetric water-holding capacity (vwc). The soil, which had been used for winter wheat and barley cropping, was sieved (2.55-mm mesh), loose root fragments were removed, and the soil was used immediately or after storage at 4°C for a maximum of 1 month. Soil packing was kept as even as possible along the tube by frequent tapping of the tube on a bench top. Immediately after soil packing, a 10-cm Rhizon Sampler[®] was pushed vertically down into the soil so that it was completely buried (Figure 1). Carbon substrates were perfused into the soil through three lengths of sterile Santoprene[®] (Exxon Mobil Corporation, Ohio, USA) tubing (SCO320, orange-blue, i.d. 0.25 mm) placed, under sterile conditions, in a glass Wheaton crimped-neck bottle containing 100 ml of treatment solution and sealed with Parafilm. Tubes were attached

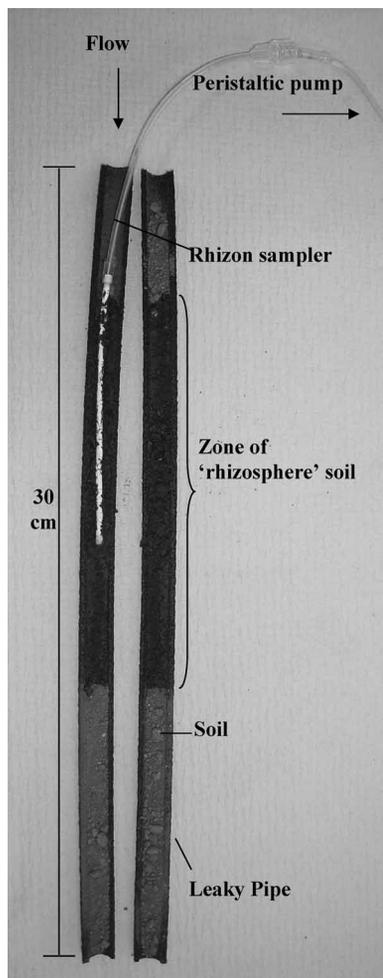


Figure 1. Photograph of rhizocosm, bisected to illustrate its construction.

to the Rhizon Samplers[®], and substrates were pumped through the rhizocosm (Watson Marlow 205S peristaltic pump) at a flow rate of approximately 3 ml d^{-1} . Rhizocosms were incubated in a phytotron (Sanyo Fi-totron PG660) at 12°C for 10 days.

To assess potential advantages of the perfusion rhizocosms, an alternative system was constructed by filling a sterile universal vial with 10 g fresh weight sterile soil. This mass is equivalent to the harvest mass of soil from the Leaky Pipe[®] rhizocosm. Glucose (50 mM carbon) was added in enough water to bring the soil to approximately the same vwc as described previously. The vials were then incubated for 10 days under the same conditions.

Microbial Inocula

Pseudomonas fluorescens cultures (Table 1) were maintained on LB agar plates. Single colonies were placed in sterile universal vials containing 10 ml LB medium at 25°C and incubated on an orbital shaker (250 rpm for 14 h); quarter-strength Ringer's solutions washed stationary-phase cells were used to inoculate soil. One hundred μl of inoculum was pipetted onto the barrel of a rhizon sampler immediately prior to placement in the soil-filled rhizosphere chamber (Figure 1), giving an initial rhizosphere starter inoculum of approximately 10^8 cfu g^{-1} soil. The *P. fluorescens* P60rr strain used for the inoculation-recovery experiment (Raaijmakers, pers. comm.) was first plated from glycerol stocks onto selective medium comprising LB agar plus rifampicin ($100 \mu\text{g mL}^{-1}$). Colonies were then subcultured onto LB agar plates for routine use.

Carbon Substrates and Signals

Rhizocosms were supplied with a sterile, basal carrier solution of ammonium nitrate (NH_4NO_3) and dipotassium phosphate (K_2HPO_4) supplemented with C-substrate and/or signal molecules at final molar proportions of C-N-phosphorus (P) of 10:1:0.1. Treatments were as follows: water, glucose (50 mM), glucose (25 mM) + fructose (25 mM), glucose (37.5 mM) + potassium acetate (12.5 mM), glucose (43.75 mM) + proline (6.25 mM), glucose (49.9 mM) + C_4 HSL (0.1 mM), and glucose (49.9 mM) + 2(5H)-furanone (0.1 mM).

Extraction of DAPG from Soil

Soil (10 g) was harvested from the volume surrounding the rhizon sampler in each rhizocosm and placed in a Wheaton vial, which was sealed with silicone-lined lids after addition of 13 mL 80% acetone (acidified to pH 2.0). The vials were then shaken on an end-over-end shaker for 2 h at room temperature, left stationary for 15 min to allow settlement, and filtered into a clean Wheaton vial through glass-fiber filter paper (Whatman GF/C). The acetone was then evaporated off, and the

Table 1. DAPG producing *Pseudomonas fluorescens* strains or isolates screened

Strain/isolate	Reported host	Reference
P60rr (1)	Wheat	Raaijmakers (pers. comm.)
Pf5 (2)	Cotton	Howell and Stipanovic, 1979
F113 (3)	Sugar beet	Shanahan et al. 1992
Q2-87 (4)	Wheat	Vincent et al. 1991
CHA0 (5)	Tobacco	Keel et al. 1992

remaining liquid was placed in a solid-phase extraction Phenomenex C18-E cartridge (pre-equilibrated with 4 mL methanol followed by 4 mL deionized water) with a bed volume of 100 mL. A further 4 mL of methanol was added and pulled through the cartridge under vacuum. DAPG was eluted under vacuum with 4 mL 100% methanol, and 1 mL was taken for HPLC (ThermoSeparation Products) analysis. The HPLC gradient started at 60:40 (acetonitrile–water) with a 5 min ramp to 90:10, returning to 60:40 over a further 5 min. The DAPG was detected at 270 nm by the peak retention time (typically 4.3 min). A five-step dilution series of DAPG in methanol (100 to 0.01 $\mu\text{g mL}^{-1}$) was run as a standard calibration, and the regression coefficient (typically $y = 758172x$, $R^2 > 0.999$) was used to quantify sample DAPG.

To determine the extraction efficiency for the total protocol, replicate 10-g samples of soil were spiked with DAPG (Toronto Research Chemicals, Canada) at 10, 1, or 0.1 $\mu\text{g DAPG g}^{-1}$ fresh weight soil. These were then extracted following the protocol and run against a DAPG dilution series. Extraction efficiency from solid-phase extraction cartridges was assessed by determining DAPG after addition of similar quantities of DAPG to water in the extraction procedure (soil water plus extraction water), followed by extraction using the solid-phase cartridges as described previously.

RESULTS

The survivorship of an initial inoculum of *P. fluorescens* in contrasting microcosm designs is shown in Figure 2. Figure 3 presents data on DAPG extracted from the contrasting rhizocosm designs. A one-way ANOVA of the P60rr data revealed that the Leaky Pipe[®] + P60rr + C treatment contained significantly more DAPG than other treatments ($P = 0.33_{(F3, 11)}$) with the Leaky Pipe[®]–C containing the same amount of DAPG as the universal + C treatment. The final P60rr treatment (universal–C) contained the least DAPG.

Figure 4 presents data on recovery of DAPG (expressed as ng g^{-1} soil dry weight) from soils inoculated with several *Pseudomonas* strains and challenged with different C substrates and substrates plus signal molecules. The pseudomonads challenged with the control treatment of water only did not produce detectable DAPG and were therefore not included in Figure 4. Addition of simple reducing sugars (glucose and glucose + fructose) elicited a positive response from all strains and the strongest response from strain Q2-87 (Figures 4a and 4b) treatments, which was two orders of magnitude higher than other strains. Addition of glucose and signal molecules (Figures 4c and 4d) elicited a mixed response from the strains, ranging from undetectable and trace concentrations to a single high response to furanone for one of the Q2-87 replicates (indicated by *). Figures 4e and 4f show the response to increasingly complex C additions,

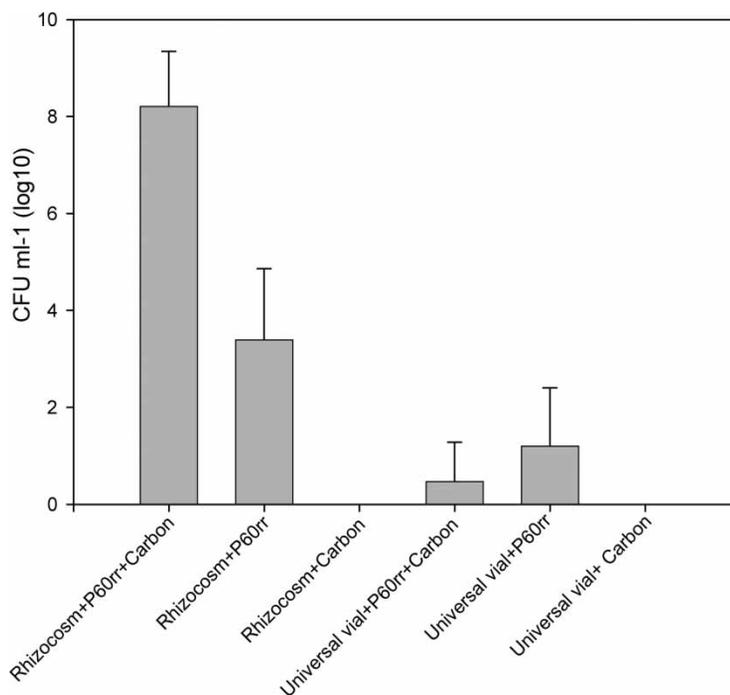


Figure 2. Survivorship of an initial inoculum of $9 \log_{10} \text{ ml}^{-1}$ *P. fluorescens* P60rr harvested from contrasting microcosms after 10 days. No inoculum were used in the negative controls (Leaky Pipe[®] + carbon and the Universal + carbon treatments). Error bars show standard error, $n = 3$.

glucose plus acetic acid (as acetate) and glucose plus proline. Only F113 showed a positive response to organic acid and glucose (Figure 4e), whereas only PF5 showed a response to amino acid amendment (Figure 4f).

Because of the polar nature of DAPG and thus its potential to bind to charged surfaces, it was necessary to determine the extraction efficiencies separately for the composite parts of the protocol: that of DAPG from the C-18E cartridge and that of the soil plus the C-18E cartridge. The DAPG recovery from the extraction cartridges was 100%. The total DAPG recovery from the soil was 38% (± 6.5 SE). The DAPG extraction data were corrected accordingly and expressed as ng g^{-1} soil.

DISCUSSION

The aim of this study was to design a rhizocosm with a manageable yet realistic soil environment within which to work and relatively homogenous

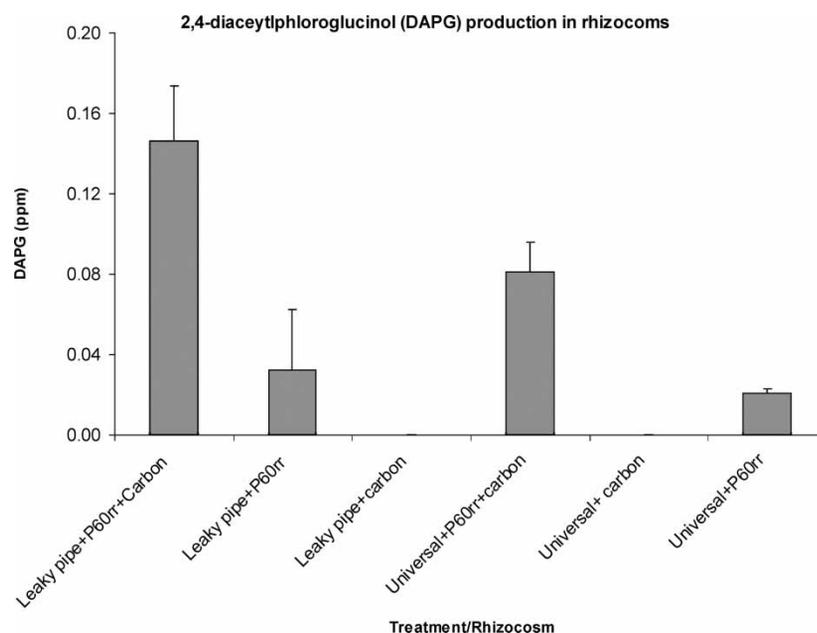


Figure 3. DAPG extraction from 10 g soil harvested from contrasting microcosms after 10 days. Error bars show standard error, $n = 3$.

soil volumes for further analyses. A rifampicin resistant *P. fluorescens* strain (P60rr) isolated from the wheat rhizosphere (Raaijmakers, pers. comm.) was used to inoculate sterile soil subjected to treatments designed to test the utility of a perfusing rhizocosm against a more traditional incubation approach. Recovery of P60rr served as a useful comparison of microbial population performance using the two microcosm design approaches. A large population of P60rr was recovered from the Leaky Pipe[®] rhizocosms. This is in contrast to the residual populations extracted from the universal vial, closed-batch microcosms. Methanol-extractable DAPG was found in both Leaky Pipe[®] rhizocosms and universal microcosms. However, the Leaky Pipe[®] design contained significantly more DAPG.

The new rhizocosm design offers a powerful system for the study of rhizosphere microbial populations in soil. The design enabled development of a large and active population from a pseudomonad inoculum, as well as the production of significantly more DAPG, than closed-batch systems. As natural rhizospheres maintain large microbial communities, we suggest that the Leaky Pipe[®] rhizocosm design more accurately mimics these than the more traditional, closed-batch design.

The novel rhizocosm has wide potential for rapid and high-throughput in situ screening of soil–microbe interactions. Its applicability is demonstrated using the exemplar of antibiotic production by soil organisms.

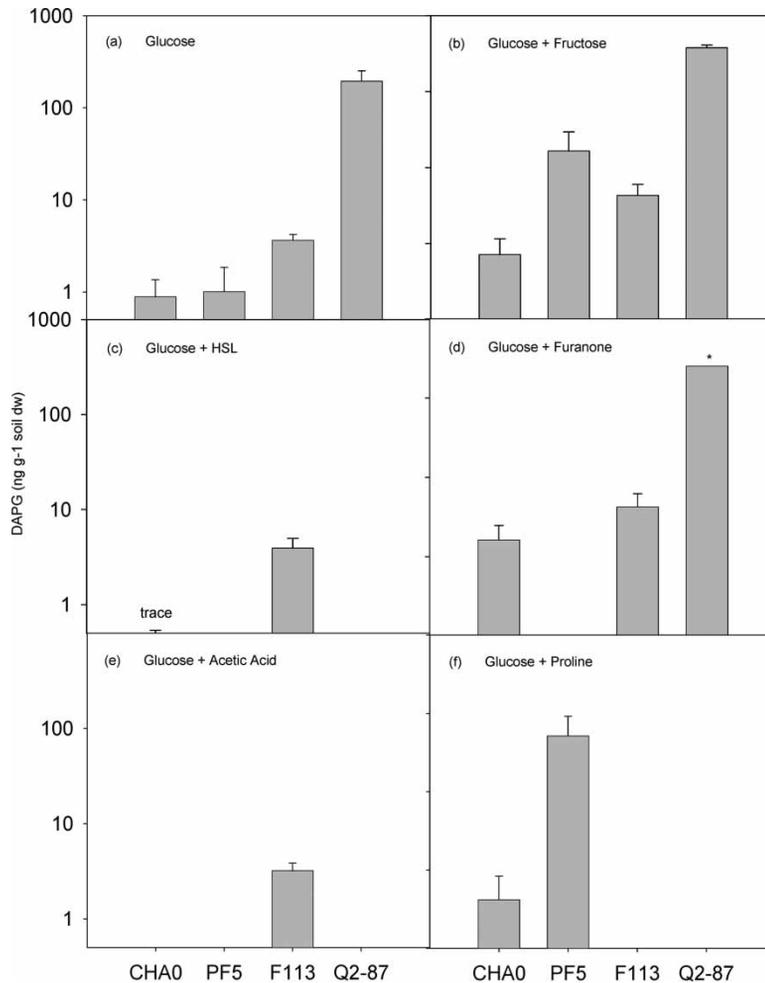


Figure 4. DAPG (ng g⁻¹ dry weight soil) extracted from soil inoculated with each of the four *P. fluorescens* strains (Table 1) and simulated rhizosphere carbon compounds. Error bars show 1 sem, n = 3; * indicates a single result.

In contrast to the traditional single-pulse method of applying C to soils, a peristaltic pump was used to supply C to soil continuously. The continual-supply method was successfully used by Griffiths et al. (1998) in their assessment of microbial response to substrate loading rates, although it has been modified here, replacing a passive wick with substrate supplied under positive pressure. It can be argued that this is unrealistic as root exudation is passive and therefore controlled by concentration gradients and membrane permeability coefficients (D. Jones, pers. comm.). However, this method, linked with equal outward flow from the whole surface area of the Rhizon

sampler and the capillary effects of the rhizocosm wall, permits a homogenous and controlled flow that equally affects soil volumes. When destructively sampling the rhizocosms, the volume of affected soil is clearly demarked (Figure 1).

The challenge of determining which components of rhizosphere C flow optimally induce antibiotic synthesis has received relatively little attention, despite the fact that soil C is ultimately plant-derived. Known antibiotic-producing strains of pseudomonads were challenged with differing simulated rhizosphere organic C composition, although glucose was the main component because it has been shown to approximate to that of early growth stage of wheat (Yeomans et al. 1999). The added C was calculated to support populations of approximately 5×10^9 cells g^{-1} soil and was supplemented with N and P to ensure that there was no nutrient limitation. It is well established that rhizosphere bacterial populations are strongly regulated by microfaunal grazers such as protozoa and bacteriophagous nematodes (Bonkowski 2004), although their effects were not determined for this set of experiments. However, the design of the rhizocosm lends itself to such multitrophic experiments.

The soil itself did not appear to carry a significant background population of DAPG producers. Without the addition of an initial inoculum, no DAPG was recovered, although soil extractions and PCR using *phlD*-specific oligonucleotides revealed that the gene was present (data not shown). This was initially an unexpected finding because DAPG-producing populations are present in most soils (McSpadden Gardner and Weller 2001; Picard et al. 2000; Raaijmakers, Weller, and Thomashow 1997; Weller and Cook 1983) and the introduction of readily assimilable substrates such as glucose should have stimulated microbial growth sufficiently for DAPG production. However, Shanahan et al. (1992) demonstrated that DAPG production was temperature dependent, with an optimum at 12°C, as chosen for our study, but growth rates of indigenous pseudomonads will be low at this temperature. As conditions in this study mimicked rhizosphere C flow from young plants (i.e., majority glucose) (Yeomans et al. 1999), this can also explain a lack of DAPG in uninoculated soils, but it would be simple to modify the assay to take account of these factors.

Different model rhizosphere C chemistries affected the production of DAPG by bacteria. Simple reducing sugars elicited the greatest overall response. The amendment of glucose with either acetate or proline resulted in inhibition of DAPG production for all strains except for F113 and PF5 (Figures 4e and 4f). Interestingly, both organic and amino acids have previously been shown to weakly elicit biosynthesis of DAPG in strain F113 (Shanahan et al. 1992). Notably, DAPG was found in soils inoculated with F113 in all the treatments, with the exception of glucose + proline (Figure 4f), in contrast to the findings of Shanahan et al. (1992) and Duffy and Defago (1999) that F113 cultures when supplied with sole C sources did not respond to glucose.

The extraction protocol used here was amended from existing protocols (Shanahan et al. 1992; Bonsall, Weiler, and Thomashow 1997; Raaijmakers, Bonsall, and Weller, 1999). The approximate 40% extraction efficiency from soil is lower than the 60% maximum quoted by Bonsall, Weller, and Thomashow (1997), although the number of extraction steps is significantly reduced. This is cost-effective and advantageous for high sample throughput. The system is currently being used to explore the retention of DAPG within different fractions of the soil matrix.

The addition of very low (μM) concentrations of signal molecules to glucose dramatically altered the DAPG response. The HSL was added at approximately 10 times the effective concentration (50% inhibition) found for this compound in liquid cultures (Saleh et al. 1999) to overcome soil quenching effects, such as adsorption to humic and clay fractions. The short-chain HSL treatment correlated with suppression of DAPG production in every strain tested except F113, which produces three HSLs (Laue et al. 2000), ranging from short-chain C_6 to long-chain C_{14} , and it is likely that the C_4 HSL used in this study did not affect it. The CHA0 was not expected to be affected by the presence of the HSL as it has been recently reported that antibiotic production by this strain is not under HSL control (Kay, Dubuis, and Haas 2005). It is interesting to observe though, that this short-chain HSL appeared to have a dramatic effect on DAPG production.

Nonhalogenated furanones are produced by many higher plants (Slaughter 1999), and 2(5H)-furanone has been shown to exhibit antifungal activity (Paulitz et al. 2000), as well as quenching quorum-sensing behavior in *Escherichia coli* pSB1075 (Budde, Standing, and Killham, pers. comm.). Furanone completely inhibited antibiotic synthesis in two strains and partially in another, opening exciting possibilities for further study. Similar to other signal/signal mimic molecules, its effects are dose dependent, and the possibility cannot be excluded that the effective concentration within the soil was not the bioactive optimum.

The potential for manipulating rhizosphere biology for crop protection relies on a fuller understanding of the triggers and mechanisms of bacterial gene expression. One promising route to this is to investigate the effects of substrate/signal compounds that are naturally found in the rhizosphere (i.e. plant root exudates). The main advantage of the novel rhizocosm system over traditional, closed-batch systems is that it presents a convenient way to create controlled, relatively homogenous, simulated rhizospheres in large enough quantities to be amenable to multidimensional studies. The ease of construction and lack of expense make this setup ideal for quick and rapid in situ screening of bacterial inocula response to various scenarios. This method offers the potential for utilizing the large number of community analysis molecular tools currently available, in particular to screen for indigenous populations in different soils with differing C additions. We are currently using the system to explore in situ relationships between wheat, DAPG-producing pseudomonads, and the fungal pathogen *Gaeumannomyces graminis* var. *tritici*.

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