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Quantification of 2,4-Diacetylphloroglucinol Produced by Fluorescent *Pseudomonas* spp. In Vitro and in the Rhizosphere of Wheat

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The broad-spectrum antibiotic 2,4-diacetylphloroglucinol (Phl) is a major determinant in the biological control of a wide range of plant diseases by fluorescent *Pseudomonas* spp. A protocol was developed to readily isolate and quantify Phl from broth and agar cultures and from the rhizosphere environment of plants. Extraction with ethyl acetate at an acidic pH was suitable for both in vitro and in situ sources of Phl. For soil samples, the addition of an initial extraction step with 80% acetone at an acidic pH was highly effective in eliminating polar organic soil components, such as humic and fulvic acids, which can interfere with Phl detection by high-performance liquid chromotography. The efficiency of Phl recovery from soil by a single extraction averaged 54.6%, and a second extraction added another 6.1%. These yields were substantially greater than those achieved by several standard protocols commonly used to extract polar phenolic compounds from soil. For the first time Phl was isolated from the rhizosphere environment in raw soil. Following application of *Pseudomonas fluorescens* Q2-87 and the Phl-overproducing strain Q2-87(pPHL5122) to the seeds of wheat, 2.1 and 2.4 μg of Phl/g of root plus rhizosphere soil, respectively, were isolated from wheat grown in a Ritzville silt loam; 0.47 and 1.3 μg of Phl/g of root plus rhizosphere soil, respectively, were isolated from wheat grown in a Shano silt loam. However, when the amount of Phl was calculated on the basis of cell density, Q2-87(pPHL5122) produced seven and six times more antibiotic than Q2-87 in Ritzville silt loam, and Shano silt loam, respectively.

Phloroglucinol antibiotics are phenolic bacterial and plant metabolites with antifungal, antibacterial, antiviral, anthelmintic, and phytotoxic properties (19). The broad-spectrum antibiotic 2,4-diacetylphloroglucinol (Phl) is a major determinant in the biocontrol activity of the plant growth-promoting rhizobacteria (PGPR) (24) *Pseudomonas fluorescens* CHA0 against black root rot of tobacco (*Thielaviopsis basicola*) and take-all of wheat (*Gaeumannomyces graminis* var. *tritici*) (8), *P. fluorescens* Q2-87 against take-all of wheat (7, 22), and *P. fluorescens* F113 against damping off of sugar beet (*Pythium ultimum*) (5, 17).

A 4.8-kb DNA fragment containing a portion of the Phl biosynthetic locus from strain Q2-87 (1, 22) was used as a hybridization probe to demonstrate that the *phl* genes are conserved in all strains within a worldwide collection of Phl producers, many of which are known biocontrol agents. Furthermore, Phl producers appear to be especially abundant in soils that are naturally suppressive to certain diseases (9). Harrison et al. (7) estimated that at least 20% of the fluorescent pseudomonads isolated from *G. graminis* var. *tritici*–infected roots grown in a take-all-suppressive soil from Quincy, Wash., had a phenotype that is characteristic of Phl producers. Of the *Pseudomonas* strains isolated from wheat roots grown in soils suppressive to *T. basicola* from the Morens region of Switzerland, 23% contained the Phl biosynthesis locus (9).

As part of our continuing effort to understand the role of Phl in biological control of root diseases by introduced or naturally occurring PGPR, we developed a protocol, drawing from several reported methods (8, 13, 22), that can readily be used to isolate and quantify Phl from agar and broth cultures and from the roots and rhizospheres of plants. We then demonstrated for the first time the production of Phl in a natural rhizosphere environment, using wheat treated with *P. fluorescens* Q2-87 and grown in raw soils.

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MATERIALS AND METHODS

Organisms and culture conditions. A rifampin-resistant derivative of *P. fluorescens* Q2-87 (14, 22) was used as the wild-type strain. *P. fluorescens* Q69c-80(pVSP41) (6) is a rifampin-resistant derivative of Q69c-80 (7, 14) containing the plasmid pVSP41 (21). Strains Q2-87(pPHL5122) and Q69c-80(pPHL5122) are rifampin-resistant derivatives of Q2-87 and Q69c-80 carrying pVSP41 with the 6.5-kb Phl biosynthesis locus (1).

Phl production by these bacteria was assayed in broth and on agar plates. In broth studies, 4 ml of yeast malt (YM) broth (1) in 15-ml test tubes was inoculated and incubated at an angle on a rotary shaker (300 rpm) for 60 h either at room temperature (22 to 24°C) or in an incubator (27°C). In agar plate studies, petri plates (100 by 15 mm) filled with 28 ml of YM agar were inoculated by spreading 130 μl of an overnight culture in YM broth onto the surface of the agar with a glass rod. Plates were incubated at room temperature or at 27°C for 60 h.

Seed treatment. Bacterial cultures used for seed treatments were grown as a lawn on King's medium B (KMB) (11) in standard petri dishes. After 60 h at 28°C, plates were flooded with 0.1 M phosphate buffer, pH 7.2. Cells were scraped into a centrifuge tube and washed twice by centrifugation to remove residual metabolites. Sedimented bacteria were mixed with 0.5% (wt/vol) methylcellulose and applied to wheat seeds (cv. Penawawa) (20). Typically, three plates of bacteria and 3.4 ml of a methylcellulose suspension were used to treat 20 g of seed. Coated seeds were dried under a stream of sterile air. Control seed received no treatment.

Soils. Studies of Phl production in soil used a Ritzville silt loam (RSL) (34.8% sand, 2.8% clay, and 62.4% silt; pH 8.0) collected from the Washington State University Dryland Field Station near Lind, Wash. A Shano silt loam (SSL) (46.8% sand, 4.8% clay, and 48.4% silt; pH 6.8) was collected from a site adjacent to an apple orchard near Quincy, Wash. Neither soil had been cropped previ-

ously, and both supported native vegetation. The soils were collected from the upper 30 cm of the soil profile, air dried, passed through a 0.5-cm mesh screen, and stored in plastic buckets prior to use.

Wheat seedlings. Plastic pots (diameter, 16.5 cm) were filled with 1,400 g of soil, and 400 ml of dilute (1:3, vol/vol) Hoagland's solution (macroelements only) (14) was added 1 day before planting. Immediately before planting, another 100 ml of dilute Hoagland's solution was added to each pot, and then 65 wheat seeds were sown in each pot and covered with 300 g of soil. Each pot was placed in a clear plastic bag, and the top of each bag was closed with a rubber band to prevent drying of the soil surface. Pots remained in the laboratory for 3 days and then were incubated in a growth chamber or a greenhouse (18 to 28°C) for an additional 8 days with the tops of the plastic bags open. Each pot received 100 ml of dilute Hoagland's solution every 3 days after the plastic bags were opened. At harvest, the soil and roots were removed from each pot. Individual plants were carefully separated, and excess soil was shaken from each root system, leaving the adhering rhizosphere soil. The remnant of the seed was removed from each plant, and the roots were severed from the rest of the plant at the crown.

Root colonization. Population sizes of introduced bacteria on seeds and roots were determined essentially as previously described (20, 23). Immediately after planting, five treated seeds were shaken in a 250-ml flask with 50 ml of 0.1 M phosphate buffer, pH 7.2, on a rotary shaker (250 rpm). Aliquots (0.1 ml) of appropriate serial dilutions were plated on KMB. At harvest, roots from one or two plants from the same pot were weighed and were macerated in 20 ml of phosphate buffer with a mortar and pestle, and aliquots of appropriate dilutions were plated onto KMB amended with 100 μg each of rifampin and cycloheximide per ml. Plates were incubated at 27°C, and colonies were counted after 48 h.

Antibiotic isolation and detection. Broth cultures including bacterial cells were acidified with 44 μl of 10% trifluoroacetic acid (TFA) to pH 2.0 and then extracted twice with 10 ml of ethyl acetate (90% extraction efficiency). The organic phase containing Phl was evaporated to dryness and suspended in 3.0 ml of 35% acetonitrile (ACN) with 0.1% TFA. These crude extracts were filtered through 0.20-μm syringe filters (Acrodisc) and fractionated by C_{18} reverse-phase high-performance liquid chromatography (HPLC) (Waters NOVA-PAK C-18 Radial-PAK cartridge, 4 μm, 8 by 100 mm) with a 55-μl sample injection.

The Waters HPLC system consisted of a 710B WISP, 510 pumps, and a 680 automated gradient controller with a 990 photodiode array detector. Solvent conditions included a flow rate of 1.0 ml/min with a 2-min initialization at 10% ACN–0.1% TFA followed by a 20-min linear gradient to 100% ACN–0.1% TFA. HPLC gradient profiles were monitored at the spectral peak maxima (270 and 330 nm) that are characteristic of Phl in the designated solvent system (Fig. 1). Phl eluted at a retention time of 17.02 min in approximately 78% ACN–0.1% TFA. Standard curves were generated by spiking cultures of the Phl-non-producing strain Q69c-80(pVSP41) with known concentrations of Phl (20 to 120 μg, taken from a 1-mg/ml stock solution of Phl in methanol) and subjecting the mixture to the extraction procedure described above. Seven point standard curves were found to be completely linear and reproducible, yielding correlation coefficients ranging from 0.9994 to 0.9997 with a 1-μg detection limit.

The following changes in this HPLC protocol lowered the detection limit of Phl to 400 ng. After extraction and drying, samples were dissolved in 270 μl of 35% ACN–0.1% TFA and spun for 10 min in an Eppendorf 5415 centrifuge at 14,000 rpm. The supernatant was removed, and 200 μl of it was injected into the HPLC. After a 2.0-min initialization at 10% ACN–0.1% TFA, curve profile 5 from the Waters 680 automated gradient controller was used for a 150-min gradient to 100% ACN–0.1% TFA. Phl eluted at a retention time between 19 and 20 min. The seven-point standard curves (400 to 1,000 ng) had a correlation coefficient of 0.9958.

To isolate Phl from YM agar cultures, four agar discs (1.6 cm in diameter) were cut with a cork borer from a plate. Four discs were equivalent to approximately 4 ml of medium, the volume used in the broth studies. Each of the four discs was sectioned into four equal pieces, and all of the pieces were extracted for 2 h by shaking (200 rpm) in 10 ml of 80% acetone. The extract then was filtered through cheesecloth, evaporated in a fume hood to 4 ml of water, acidified to pH 2.0 with 44 μl of 10% TFA, and extracted twice with 10 ml of ethyl acetate (52% extraction efficiency). Samples were dried, dissolved in 3.0 ml of 35% ACN–0.1% TFA, and filtered through 0.20-μm syringe filters, and 110 μl was injected and eluted as described above for broth extraction with a 1-μg detection limit. Standard curves were generated by growing the non-Phl-producing strain Q69c-80(pVSP41) on YM agar. After agar discs were cut, sectioned, and placed in 80% acetone, known concentrations of Phl (20 to 120 μg) were added, and the mixtures were subjected to the extraction procedure as described above. Seven-point standard curves (20 to 120 μg) yielded a correlation coefficient of 0.9834 and a detection limit of 1 μg.

To isolate Phl from the rhizosphere environment, wheat roots with adhering rhizosphere soil and without remnants of seeds (25 g [fresh weight] from approximately 50 seedlings) were mixed with 30 ml of 80% acetone in a flask. The mixture was acidified to pH 2.0 with 340 μl of 10% TFA, and then shaken (200 rpm) for 2 h at room temperature. Samples then were suction filtered through a Buchner funnel lined with Whatman no. 1 filter paper, and the filtrate was centrifuged at 9,500 rpm for 30 min at 4°C in a Beckman J2-21M centrifuge with a JA-17 rotor. The supernatant was decanted, evaporated to 4 ml of water, acidified to pH 2.0 with 10% TFA, extracted twice with 10 ml of ethyl acetate, and dried as described above. Before injection, extracts were suspended in 1 ml of 35% ACN–0.1% TFA, precipitated overnight at −20°C, and then centrifuged in an Eppendorf 5415 centrifuge at 14,000 rpm for 20 min at 3°C. The supernatant was removed, and 55 μl was injected. Seven-point standard curves (20 to 120 μg) yielded a correlation coefficient of 0.9978 with a 1-μg detection limit.

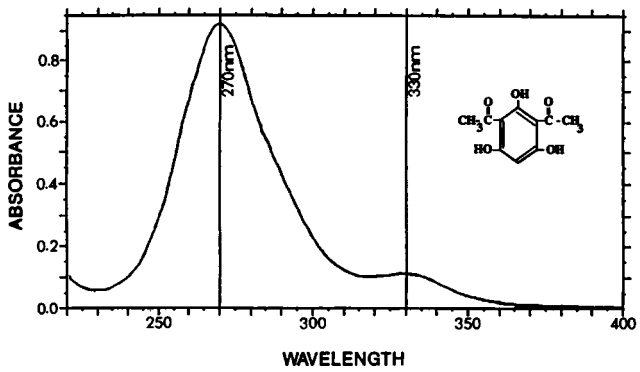

FIG. 1. UV-visible spectrum and structure of Phl.

Preparation of roots spiked with Phl. Because the method of spiking roots could affect extraction efficiency, three methods of adding Phl to roots of wheat grown in SSL were tested. In method 1, 15 g of wheat roots with adhering rhizosphere soil (from 11-day-old seedlings) was placed in a petri dish. Phl (40 μg taken from a 1-mg/ml stock solution in methanol) in 50 ml of acetone was evenly dispersed over the roots, and the roots were air dried before extraction with 30 ml of 80% acetone. In method 2, 40 μg of Phl from the stock solution was added directly to the 15 g of roots with adhering soil immediately before extraction with 80% acetone. In method 3, 30 ml of 80% acetone was added to 15 g of roots with adhering soil and then 40 μg of Phl from the stock solution was added.

Comparison of acetone extraction with extraction procedures for plant phenolic acids from soil. Roots with adhering rhizosphere soil (15 g) from wheat grown in SSL were mixed with 30 ml of either 80% acetone, 250 mM EDTA at pH 7.0, 0.5 M NaOH, or 0.5 M sodium acetate (2, 4, 10). Each sample was amended with Phl (20 or 40 μg) by method 3 and then shaken for 3 h at 200 rpm. Samples were suction filtered, and the filtrates were centrifuged at 9,500 rpm for 30 min in a Beckman J2-21M centrifuge with a JA-17 rotor as described above. The organic filtrate from the 80% acetone extraction (organic fraction) was processed as described above. The aqueous filtrates from the EDTA, NaOH, and sodium acetate extractions were titrated to pH 2.0 with HCl and extracted twice with 30 ml of ethyl acetate (aqueous fractions). Fractionation by HPLC was as described above.

For each sample, the roots and soil after filtration and the soil pellet after centrifugation were combined and extracted a second time in 30 ml of 80% acetone. Samples were shaken for 2 h in 80% acetone at an acidic pH, evaporated to 4 ml of water, titrated to pH 2.0 with 10% TFA, extracted twice with ethyl acetate at an acidic pH, and analyzed by HPLC (organic fractions).

Statistical analysis. Treatments were arranged in a randomized complete block design. All experiments were repeated at least once, and representative results are reported here. Population densities of bacteria on seeds and roots were compared by analysis of variance.

RESULTS

Production of Phl in vitro. Phl was readily isolated from all the strains cultured on YM broth or agar, except from the control, Q69c-80(pVSP41) (Table 1). Regardless of whether Phl production was expressed as micrograms per milliliter of culture, total micrograms, or micrograms per A_{600} unit, all strains produced more Phl on agar plates than in broth. The difference was greatest with Q2-87, which produced 47.7 or 60.3 times more Phl (based on micrograms per A_{600} unit) on agar plates than in broth at room temperature (22 to 24°C) or 27°C, respectively (Table 1). Furthermore, strains Q2-87 and Q69c-80(pPHL5122) produced approximately twofold more Phl at 27°C than at room temperature in both broth and agar cultures. Temperature had little or no effect on Phl production by Q2-87(pPHL5122). In both broth and agar media and at both temperatures, Q2-87(pPHL5122) produced substantially more Phl than the wild-type Q2-87. Across temperatures and media the increase ranged from 12- to 278-fold. In both media

TABLE 1. Production of Phl by *Pseudomonas* strains Q2-87, Q2-87(pPHL5122), and Q69c-80(pPHL5122)

Strain[a]	Temperature[b]	Medium	Phl concn (μg/ml of culture)[c]	Phl amt (total μg)[c]	A_{600}[d]	μg/A_{600}
Q2-87	RT	Broth	0.63	1.9	1.18	1.7
		Agar	32	96	1.19	81
	27°C	Broth	0.63	1.9	0.60	3.3
		Agar	65	194	0.98	199
Q2-87(pPHL5122)	RT	Broth	112	336	0.70	473
		Agar	195	585	0.50	1,000
	27°C	Broth	89	266	0.48	560
		Agar	181	544	0.48	1,142
Q69c-80(pPHL5122)	RT	Broth	1.5	4.5	1.31	3.4
		Agar	3.5	10	1.17	8.9
	27°C	Broth	5.2	16	1.09	14
		Agar	6.0	18	1.13	16

[a] The control strain Q69c-80(pVSP41) (data not shown) produced no detectable Phl in YM broth or agar.
[b] Room temperature (RT) was 22 to 24°C.
[c] Values are the means of at least three replicates.
[d] Cell density.

and at both temperatures, Q2-87(pPHL5122) attained a lower cell density and died more rapidly than the wild type (data not shown). The reason for this is not known and is under investigation.

Extraction of Phl from spiked roots. The method of spiking wheat roots with Phl had no effect on the extraction efficiency. For example, of the 40 μg of Phl added, 49.2, 48.3, and 50.3% were recovered from roots treated by methods 1, 2, and 3, respectively. Method 3 was adopted in subsequent experiments because it was the easiest.

Effect of extraction method on recovery of Phl from roots. With only one extraction, 80% acetone recovered an average of 54.6% (range of 53.0 to 66.5%) of the Phl added to the roots. A second acetone extraction recovered an additional 6.1% (range of 5.6 to 6.9%) of the added Phl. In contrast, yields from aqueous extractions with 0.5 M sodium acetate, 250 mM EDTA (pH 7.0), and 0.5 M NaOH were 7.4, 3.0, and 0.0%, respectively (Table 2). When the roots and soil (after filtration) and the soil pellets were combined and extracted with 80% acetone, yields from the sodium acetate, EDTA, and NaOH treatments were 3.3, 5.0, and 0.0% (Table 2).

TABLE 2. Comparison of the recovery of Phl from roots with adhering soil by different extraction methods[a]

Extraction method	% Yield		
	Aqueous fraction	Organic fraction	Total (aqueous + organic fractions)
80% acetone (1st extraction)	NA[b]	54.6	54.6[c]
80% acetone (2nd extraction)	NA	6.1	6.1[d]
0.5 M sodium acetate	7.4	3.3	10.7[e]
250 mM EDTA, pH 7.0	3.0	5.0	8.0[e]
0.5 M NaOH	0.0	0.0	0.0[f]

[a] Plants were grown in SSL.
[b] NA, not applicable.
[c] Mean from seven separate extractions. Range = 53.0 to 66.5%.
[d] Mean from five separate extractions. Range = 5.6 to 6.9%.
[e] Value is the greater from two separate extractions.
[f] Value from one extraction.

Production of Phl in soil. In all experiments, the population sizes established on the surfaces of wheat seeds were significantly ($P = 0.05$) greater for Q2-87 than for Q2-87(pPHL5122) (Table 3). This was because Q2-87(pPHL5122) produced less cell mass in both agar and broth cultures (Table 1) and survived poorly on the seed compared to the wild-type strain (Table 3). Furthermore, in the rhizosphere environment, the population sizes of Q2-87(pPHL5122) were significantly smaller than those of Q2-87 for both the RSL (1.1×10^7 versus 6.4×10^7 CFU/g of root with adhering soil; $P = 0.05$) and the SSL (1.4×10^7 versus 2.9×10^7 CFU/g of root with adhering soil; $P = 0.05$). In spite of these differences in population densities, in the RSL Q2-87(pPHL5122) produced slightly more Phl than Q2-87 (2.4 and 2.1 μg/g of root with adhering soil, respectively); in the SSL Q2-87(pPHL5122) produced more than twice as much Phl as Q2-87 (1.3 and 0.47 μg/g of root with adhering soil, respectively) (Table 3). When normalized to equivalent population densities, the amount of Phl produced by Q2-87(pPHL5122) was approximately seven- and sixfold greater than that produced by Q2-87 in RSL and SSL, respectively (Table 3).

DISCUSSION

Antibiotic production is now recognized as an important mechanism by which biocontrol agents, especially PGPR, suppress plant pathogens. However, isolation from natural sources such as roots and soil has been achieved for relatively few antibiotics known to contribute to biological control (18). To our knowledge, this is the first report of the production of Phl by introduced bacteria in a rhizosphere environment in raw soil. Previously, Phl was isolated from the roots of wheat colonized by *P. fluorescens* CHA0 growing in a gnotobiotic system in which sterile artificial soil, composed of vermiculite clay, quartz sand, and quartz powder, was used as the rooting medium (8, 12). Keel et al. (8) recovered 0.04 and 0.10 μg of Phl per g of root plus rhizosphere soil from healthy roots and roots infected by *G. graminis* var. *tritici*, respectively. Using similar methods, Maurhofer et al. (12) recovered 0.25 and 0.23 μg of

TABLE 3. Recovery of Phl from the rhizosphere environment of wheat following seed treatment with *P. fluorescens* Q2-87 and Q2-87(pPHL5122)

Soil[a]	Seed treatment	CFU/seed[b,c]	CFU/g of root[c,d,e]	μg of Phl/g of root[e,f]	μg of Phl/10^7 CFU/g of root
RSL	Q2-87	4.5×10^6	6.4×10^7	2.1 (1.2–3.1)	0.33
	Q2-87(pPHL5122)	5.1×10^5	1.1×10^7	2.4 (1.2–3.7)	2.18
SSL	Q2-87	1.2×10^6	2.9×10^7	0.47 (0.25–0.80)	0.16
	Q2-87(pPHL5122)	3.3×10^5	1.4×10^7	1.3 (1.1–1.5)	0.93

[a] Each soil was used in a separate experiment.
[b] Values are means for three samples.
[c] Means for the same soil but different treatments are significantly different ($P = 0.05$).
[d] Values are means for seven and four samples for the RSL and SSL, respectively.
[e] "Root" refers to root plus adhering rhizosphere soil.
[f] Values are means from three separate extractions except for Q2-87(pPHL5122) in SSL, for which two extractions were done. Each extraction used 25 g (fresh weight) of root plus adhering soil. Values in parentheses are ranges.

Phl per g of root plus rhizosphere soil from healthy roots and from roots infected by *Pythium ultimum*, respectively. Even considering our higher extraction efficiency, we were surprised to recover substantially more Phl from roots colonized by strain Q2-87 (2.1 and 0.47 μg per g of root in RSL and SSL, respectively), because our soils were raw and contained a normal microflora, which was absent in the gnotobiotic studies with strain CHA0. Greater in situ Phl production by strain Q2-87 than by CHA0 may reflect the fact that production in vitro by Q2-87 was 14 times greater than that of CHA0 (9).

Efforts to genetically engineer *Pseudomonas* spp. with enhanced biocontrol activity have focused on increasing the amount of antibiotic produced by a strain or by transferring biosynthetic loci into heterologous strains (19, 24). It has not been clear whether there is a correlation between overproduction in vitro and in the rhizosphere environment. Maurhofer et al. (12) showed that strain CHA0/pME3090, which produced three- to fivefold more pyoluteorin and Phl than did CHA0 in vitro, overproduced these antibiotics in an artificial wheat rhizosphere and had increased biocontrol activity. Our work expands the findings obtained with strain CHA0 and demonstrates that overproduction can also occur in a rhizosphere environment in raw soil. It is notable that in the SSL and RSL, Q2-87(pPHL5122) produced six and seven times more Phl on a per-cell basis than Q2-87. We believe that the use of antibiotic-overproducing pseudomonads is a way to achieve disease control with a smaller bacterial inoculum. Reducing the dose of a PGPR required for disease suppression would lower the cost of the treatment and thus increase acceptance of the treatment by growers.

Given the importance of Phl as a mechanism of biological control, a central objective of our study was to develop an extraction protocol that could be applied easily to both laboratory and natural samples. Such a rapid method is essential to facilitate routine use of analytical techniques in concert with molecular methods to study the role of metabolites in the activity of biocontrol agents in the rhizosphere. The protocol described here, which uses ethyl acetate at an acidic pH, allows numerous samples from broth, agar, or soil to be handled quickly and easily. For agar and soil samples, we included an initial extraction with 80% acetone at an acidic pH, which is selective for nonpolar compounds and thus helps to eliminate polar contaminants such as soil humic and fulvic acids. A key step in extraction from soil samples, overnight precipitation of samples at −20°C in 35% ACN–0.1% TFA and centrifugation, facilitated further removal of humic acids and other complex organics that were carried over during the extraction. This step eliminated the need to pretreat samples on Sep-pak C_{18} cartridges before HPLC analysis, as described by Shanahan et al. (16).

The extraction efficiency of Phl from the SSL with acetone and ethyl acetate was comparable to those previously reported for Phl from an artificial soil lacking organic matter, 40 to 50% (8), and from a sandy soil spiked with Phl, 60 to 70% (15). Extraction efficiencies also greatly exceeded those achieved with standard protocols using aqueous sodium acetate, EDTA, or sodium hydroxide to extract phenolic acids from soil. Acidification to pH 2.0 maintained Phl at a neutral charge and minimized ionic interactions with charged soil particles, thus avoiding the need for salts and EDTA, which are commonly used to displace organic anions bound through cationic bridges to mineral surfaces. We avoided the use of strong acids and bases such as 0.5 N NaOH, which may hydrolyze acetyl groups from Phl, causing degradation. Furthermore, our extractants were acidified with TFA, which is more volatile than concentrated HCl (2 to 12 M). The use of HCl to acidify extractant solutions is common (2, 8, 10, 13), but HCl may become concentrated as samples are taken to dryness, thus contributing to Phl degradation.

The solvent system described in this report is a simple linear gradient that avoids multigradient or multi-isocratic steps (8, 16) and the complex solvent systems often used for the isolation of phenolic acids from soil (2, 10). In addition, we performed reverse-phase chromatography with the column at room temperature rather than at 45°C (8), as the latter temperature may promote hydrolysis of Phl. Finally, it is important to remember that reproducible quantitation of Phl at very low detection limits requires that HPLC gradient profiles be monitored at the spectral peak maxima (270 and 330 nm) that are characteristic of Phl in the solvent mixture (78% ACN–0.1% TFA) at its specific retention time (17.02 min).

It is notable that the procedure described here also is suitable for the extraction and identification of other metabolites, including phenazines, pyrrolnitrin, pyoluteorin, and indoleacetic acid (3) that have been shown to be critical to the biocontrol or growth-promoting activity of numerous PGPR strains (19). Given that the role of antibiotics in the rhizosphere is now well established, the next step is to identify the threshold antibiotic concentrations that are needed to achieve biological control and the bacterial densities required to produce these levels.

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