Herbicolin A Associated with Crown and Roots of Wheat after Seed Treatment with *Erwinia herbicola* B247

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ABSTRACT

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Wheat seeds were treated with a strain of *Erwinia herbicola* that produces herbicolin A. After 4 days' growth in a natural soil, the antibiotic was extracted from washed roots and crowns. Herbicolin A was detected in crowns and the first centimeter of roots by a thin-layer chromatography overlay inhibition assay. The chromatography technique was superior to high-performance liquid chromatography because of its sensitivity and the fact that it did not require purification of tissue extracts. Quantities of herbicolin A averaging 2.7 μg/g dry weight were detected in crowns of seedlings grown in natural field soil with an average of 3×10^9 colony-forming units per gram dry weight of tissue. The antibiotic was not detected in extracts of roots treated with a transposon mutant lacking herbicolin production. Concentration of antibiotic extracted from the tissue exceeded that which could be recovered from a bacterial culture of comparable population density to the colonizing bacteria, indicating in situ production of herbicolin. This is the first report of in situ antibiotic production in natural soil with subsequent absorption by plants.

Additional keywords: biological control.

The spatial relationships among interacting microorganisms in the rhizosphere and rhizoplane are not well defined. When studying the population dynamics of pathogens and antibiotic-producing organisms following their inoculation on seeds or roots, it is generally assumed that altered behavior of pathogens is the result of a direct and proximate relationship. However, the mechanism of action could well be indirect. For example, the production of antibiotics by a vigorously growing antagonist could change the general composition of the microflora and encourage growth of other organisms that affect the pathogen. The direct absorption of antibiotics and other substances by plant tissues

could inhibit infection by pathogens, which in turn affects tissue susceptibility.

Although there are no experimental data providing evidence that plant roots absorb antibiotics from rhizosphere microflora in natural soil, in in vitro studies plants absorbed some antibiotics, such as griseofulvin (3). Streptomycin was locally absorbed by plant cells when topically applied (7). Moreover, there is considerable indirect evidence that microbially produced metabolites are absorbed by roots. For example, the toxigenic effect of some organisms in the rhizosphere (2,13) must result from uptake of toxic metabolites. Exogenous applications of indoleacetic acid to roots and the resultant effect indicate the absorption of the compound (4); there also is indirect evidence of this happening when indoleacetic acid–producing bacteria are applied to roots (14). It is known that the exogenous application of various substances to plant parts affects plant resistance to certain organisms (16).

The hypothesis that roots can readily absorb compounds from colonizing bacteria is attractive, because it is difficult to rationalize the spatial positioning of antibiotic-producing organisms on roots with the often marked reduction of rhizosphere fungi. Bacteria colonize only relatively small surface areas of roots and occur in a lognormal frequency (5,15). Thus it is highly unlikely that application of antagonistic bacteria to plant parts results in the occupation of all infection courts available to a pathogen for ingress. It seems more likely that bacterial colonization of plant parts results in a "sphere of influence," possibly by translocation of bioactive substances from colonized sites.

Erwinia herbicola is a member of the Enterobacteriaceae, commonly isolated from soil and plants. Strains of the organism produce antibiotics effective against bacteria (9,22) or fungi (11,21). The antifungal antibiotics, herbicolin A and B, are active against a wide range of filamentous fungi, yeasts, and Mollicutes.

E. herbicola strain B247 on wheat is an attractive model for testing the hypothesis that plants can absorb antibiotics from bacteria growing in the spermosphere, rhizosphere, and perhaps the laimosphere. Wheat seedlings grown from seeds treated with *E. herbicola* and planted in soil infested with *Fusarium culmorum* were protected from seedling blight (11). Similarly, leaf rust caused by *Puccinia recondita* f. sp. *tritici* was controlled by applying culture filtrates of the bacterium or a suspension of bacteria to wheat leaves. The mechanism of control, determined by comparing a wild-type strain with an antibiosis-negative mutant, appeared to be due to the production of the antifungal agent, herbicolin A. In this report we present evidence that wheat roots and crowns absorb herbicolin A after bacterial coating of seeds. A brief report of these results was previously published (10).

MATERIALS AND METHODS

Seed treatment. *E. herbicola* B247 or its transposon mutant Tn247, containing a single Tn5 insertion and lacking the ability to produce herbicolin (11), was grown in 2% tryptic soy broth (20 g/L, pH 7.2; Difco) for 16 h at 28 C on a rotary shaker at 120 rpm. Cultures were centrifuged for 10 min at 8,000 rpm and washed once with 100 mM $MgSO_4$. Wheat seeds, *Triticum aestivum* cv. Phoenix, were surface-sterilized for 5 min in 1% NaOCl and washed three times with sterile distilled water. Seeds were immersed for 30 s in a bacterial suspension of 3×10^9 colony-forming units (cfu) per milliliter with 75 mM $MgSO_4$ and 0.25% methyl cellulose, and dried on a paper towel for 1 h at room temperature, leaving 10^6 cfu per seed.

Growth of plants. For growth chamber experiments, seeds coated with B247 or Tn247 were placed 1 cm deep into an unsterile, unamended, natural Yolo sandy loam field soil from the Davis field station, University of California, Berkeley, with a matric potential between −10 and −15 kPa. Each 200-ml plastic pot containing nine seeds was covered with a 1-cm layer of vermiculite. Treatments were randomized, and pots were incubated at 21 C and 90% RH without watering. After 4 days, the emerged seedlings were removed from the soil. In a single field trial, seeds were placed 1–2 cm deep into Yolo soil transferred to the San Jose field station. Each plot measured 25 × 25 cm and contained 300 seeds. Two plots were planted with B247-treated seeds, and two plots were planted with seeds treated only with $MgSO_4$ and methyl cellulose. The soil temperature ranged from 10–19 C at 8 a.m. to 26–32 C at 3 p.m. After 6 days, the emerged seedlings were removed from the soil.

Extraction of herbicolin from plant tissue. The seedlings were cleansed of loosely adhering soil and divided into the crown and root segments (0–1 cm, 1–2 cm, and so on). Segments of similar tissue types from approximately 300 plants were pooled, washed first under a jet of tap water (100–150 ml for crowns and 300–400 ml for roots), and then shaken in 100 ml of 10 mM phosphate buffer for 10 min at 290 rpm. Plant fractions were blotted with a paper towel and homogenized in liquid nitrogen with a mortar and pestle. The homogenate of each fraction was extracted with 20 ml of methanol for 16 h at 4 C. Plant material was separated from the methanol extract by centrifugation for 10 min at 35,000 *g*. The pellet was dried for 24 h at 110 C and weighed. The supernatant was concentrated in a rotary evaporator at 45 C to less than 1 ml, frozen at −80 C, and lyophilized overnight (≈2.6 Pa at −60 C). The resulting powder was transferred into an Eppendorf tube, and 75–150 μl of methanol was added. Tubes were sonicated (125W) in a water bath at 45 C for 30 min. After centrifugation at 35,000 *g* for 15 min, the supernatants were used for thin-layer chromatography (TLC).

High-performance liquid chromatography, TLC, and bioassay for detection of herbicolin A in plant extracts. For the TLC bioassay, 50 μl of the concentrated extract were spotted onto a TLC plate (Whatman LK6DF silica gel, 250 μm). Standards of herbicolin A (50, 100, and 200 ng) were provided by M. Greiner, University of Tübingen, Germany. Development was in chloroform/methanol/water/acetic acid (65:25:4:3, v/v) (1). Solvents were evaporated from the plate under vacuum at 10 C for 3 h. The TLC plate was placed onto 2% water agar in a baking dish and overlayered with molten 2% tryptic soy agar (pH 7.2), 3 mm thick, containing *Candida albicans* at 10^6 cfu/ml as a test organism. After incubation for 16 h at 28 C, herbicolin A was visualized on the plate as an inhibition zone in the lawn of yeast growth. Cochromatography, with a standard of 80 ng herbicolin A plus an extract from 200 crowns and roots (0–1 cm) containing approximately 250 ng herbicolin A, was used for identification of herbicolin A in the plant extract. The antibiotic was quantified by comparing the size of the inhibition zones from the standards with that from the extract (6). The detection limit for herbicolin A in the bioassay was 50 ng.

Herbicolins were identified by high-performance liquid chromatography (HPLC) with a 5-μm Dynamax C-18 reversed phase column (10 × 250 mm) (Rainin Instrument Co., Woburn, MA) fitted with a guard column. Eluates of a 95% pure sample of herbicolin A provided by M. Greiner were used for comparison. The solvents used were 1) 0.1% trifluoroacetic acid and 2) acetonitrile containing 0.1% trifluoroacetic acid. The gradients were 0–25% B in 10 min and 25–40% B in 60 min. The flow rate was 5 ml/min. Herbicolin A, detected at 210 nm, eluted at approximately 30% B. Tissue samples were freeze-dried, and the remaining solids (weighing between 230 and 600 mg) were chopped in a Polytron homogenizer (Brinkmann Instruments, Inc., Westbury, NY) with methyl chloride/methanol (4:1). The residue was shaken in 5 ml of methanol for 1 h at 120 rpm. After centrifugation at 12,000 *g*, the supernatant was passed through a C-18 Sep-Pak (Waters Assoc., Milford, MA) and eluted with 3 ml of methanol containing 0.2% formic acid. The Sep-Paks were conditioned with 5 ml of methanol followed by 10 ml of water. After vacuum centrifugation to dryness, each sample was dissolved in 100 μl of HPLC-grade water.

Determination of the recovery of herbicolin A in the extraction procedure. A 500-ng herbicolin A sample in 25 μl of water was added to the washed and homogenized crowns and root segments (0–1 cm) from 300 untreated seedlings that were extracted, concentrated, and assayed as described above.

Determination of crown and root colonization by *E. herbicola*. All phosphate buffer washes from the crowns and root segments

TABLE 1. Herbicolin A in the tissue of wheat seedlings and colonization by *Erwinia herbicola* B247 after seed coating[a]

Experiment[b]	Tissue			
	Crown		Root segment (0–1 cm)	
	Herbicolin (μg)	B247 cfu ($\pm$ SD)	Herbicolin (μg)	B247 cfu ($\pm$ SD)
First growth chamber	3.0	$(1.0 \pm 0.2) \times 10^9$	0.8	$(1.1 \pm 0.5) \times 10^8$
Second growth chamber	2.3	$(1.2 \pm 0.3) \times 10^9$	0.8	$(1.1 \pm 0.4) \times 10^8$
Field trial	2.8	$(6.8 \pm 0.6) \times 10^9$	1.2	$(4.6 \pm 1.5) \times 10^8$

[a]Wheat seeds were treated and grown as described in Materials and Methods. All values represent the amount per gram dry weight.
[b]Number of plants for each experiment and dry weights for crown and first centimeter of root are, respectively, first growth chamber: 294 plants, 193 mg (crown), and 178 mg (root); second growth chamber: 272 plants, 173 mg (crown), and 133 mg (root); and field trial: 300 plants, 177 mg (crown), and 165 mg (root).

were collected, and appropriate dilutions in buffer were plated onto King's medium B (12). Triplicate samples from each collection were analyzed for each extraction. Colonies were counted after incubation at 28 C for 24 h and are expressed as colony-forming units per gram dry weight of tissue. It was not necessary to use marked cells, since the *E. herbicola* colonies were easy to identify by their distinctive yellow color. No yellow colonies were detected in washes of untreated plants.

To determine the number of cells that remained on the plant after the washes, the crown plus root segments (0-1 cm) from 50 seedlings were homogenized in 2 ml of 10 mM phosphate buffer with mortar and pestle. The homogenate was vigorously hand-shaken in 75 ml of buffer for 1 min and then for 30 min at 290 rpm on a rotary shaker before being assayed for colony-forming units of *E. herbicola*.

Extraction of herbicolin from cells of *E. herbicola*. *E. herbicola* was grown, harvested, and washed as described for the seed treatment. A total of 10^9 cells in 1 ml of 100 mM $MgSO_4$ were extracted, and a TLC bioassay was performed as outlined for plant material.

Incubation of herbicolin A with soil. Sandy loam (60 mg, autoclaved or natural) was placed into Eppendorf tubes containing 20 μg of herbicolin A in 100 μl of double-distilled water. After 20 min at room temperature, the tubes were centrifuged at 35,000 *g* for 15 min, and 5 μl samples of the supernatant and 5 μl of herbicolin A containing 1 μg antibiotic were spotted separately onto a TLC plate for bioassay.

RESULTS

Detection of herbicolin A in plant tissue and colonization of seedlings by *E. herbicola*. Herbicolin A was detected in crown and root tissue of wheat seedlings after seeds were inoculated with *E. herbicola* B247 and grown in unamended, natural field soil in growth chamber and field experiments (Table 1). Herbicolin A was not detected when seeds were coated with only $MgSO_4$ plus methylcellulose or with the antibiosis-negative mutant Tn247. The colony-forming units per gram dry weight of tissue for B247 and Tn247 recovered from crowns were, respectively, $1.4 \pm 0.9 \times 10^9$ and $0.7 \pm 0.2 \times 10^9$; from roots, $3.1 \pm 1.6 \times 10^8$ and $4.0 \pm 1.6 \times 10^8$.

Herbicolin A was not detected in root segments located 1–2 cm from B247-treated seeds harboring $3.0 \pm 0.3 \times 10^7$ cfu/g dry wt. The R_f values of the inhibitory compounds in the extracts were comparable to those of the standards and the R_f value of 0.12 that was previously reported (1). Cochromatography of a herbicolin A standard and B247-treated plant material resulted in a single spot of inhibition. Herbicolin A was detected in the crown and the first centimeter of roots from B247-treated seeds (Table 1). In a separate experiment, sterile sand contained about 10 times more herbicolin A and colony-forming units. A total of 30 μg and $1.2 \pm 0.5 \times 10^{10}$ cfu were detected per gram dry weight of crowns for plants grown in sand versus 2.5 μg and $1.0 \pm 0.2 \times 10^9$ cfu/g dry wt for crowns of plants grown in natural field soil. After washing, the number of colony-forming units remaining during extraction was below 10^7 per 50 seedlings (crowns plus first centimeter of roots). Recovery of herbicolin A during the extraction process was approximately 70%.

In preliminary tests with 95% pure herbicolin, the limit of detection of herbicolin A using HPLC with a semipreparative column was 1 μg. This method was deemed unsuitable for quantitative detection because of its lack of sensitivity. Further, extensive purifications of tissue extracts were required prior to HPLC. By this method, herbicolin A was not detected in tissue from seeds coated with B247. In a related study, Greiner and Winkelmann (8) reported that the detection limit of herbicolin A in culture filtrates was 0.1 μg/ml using an analytical C_8 Nucleosil HPLC column (Grom, Ammerbuch, Germany) and isocratic separation in acetonitrile-phosphoric acid (0.1%)-methanol (7:3:4).

Recovery of herbicolin from cells of *E. herbicola*. No antibiotic was detected from a sample of 10^9 cells of *E. herbicola* B247 when the bacteria were grown under conditions for seed treatment.

Extraction of herbicolin A from soil. No herbicolin A activity was recovered from the aqueous supernatant after incubation of autoclaved or natural field soil with the antibiotic for 20 min.

DISCUSSION

E. herbicola colonizes crowns and roots of wheat seedlings in natural soils and produces the antibiotic herbicolin A, which is simultaneously absorbed by the plant tissue. This is the first time that such a phenomenon has been demonstrated for an antibiotic-producing microorganism. The herbicolin did not represent adsorbed material, because experiments in which the antibiotic was added to the soil suggest that it is rapidly inactivated. Similarly, the herbicolin detected did not originate from cells of *E. herbicola* remaining on plant tissues, because the amount of herbicolin extracted from 10^9 cfu grown in culture was below 50 ng. However, the extraction of plant tissue with fewer than 10^9 cfu/g dry wt yielded about 1 μg of herbicolin A per gram dry weight. We conclude that in situ production of herbicolin A occurred in the natural field soil, and that this water-soluble antibiotic was absorbed by the plant. Inactivation or adsorption of herbicolin A in the soil could explain the failure of recovery from the rhizosphere. In a related phenomenon, however, Thomashow et al (17,18) showed the presence of phenazine antibiotics produced by *Pseudomonas fluorescens* in the rhizosphere of uninfected wheat.

The concentration of herbicolin A recovered from plant tissues was correlated with the number of bacteria colonizing the tissue. The largest quantity of both herbicolin A and bacteria was found in the crown tissue. The first centimeter of root tissue, proximal to the crown, contained less than half as much herbicolin A as crown tissue, and fewer colony-forming units. The amount of herbicolin A was below the limits of detection in more basipetal regions of the root where colony-forming units were lower. Previously, the population density of B247 decreased with increasing distance from the coated seed (10).

Advantages of the TLC-bioassay for the detection of herbicolin A were its superior sensitivity over HPLC and the fact that purification of tissue extracts was not required. The authenticity of the herbicolin A identification in these studies was confirmed in four ways: 1) the R_f values of the inhibition zones from extracts

of B247 plants and that of standards were comparable to the published value (1); 2) cochromatography of tissue extracts of B247-treated plants and a standard of pure herbicolin A resulted in a single spot and a single inhibition zone; 3) no inhibition zone was found in extracts from untreated plants or plants treated with the antibiosis-negative mutant, Tn247; and 4) the inhibitory material, like herbicolin A, is active against *C. albicans*.

We believe that many substances produced by metabolizing organisms in spermospheres and rhizospheres are absorbed by plant tissues and affect both plant growth and susceptibility to plant diseases. The present findings raise the question of whether they are active inhibitors per se, or if they initiate a cascade of events resulting in induced resistance in planta (19,20). The latter may prove as important a mechanism of biological control as the direct effect of an antagonist on a pathogen.

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