Influence of Bacterial Sources of Indole-3-acetic Acid on Root Elongation of Sugar Beet

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ABSTRACT

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Indole-3-acetic acid (IAA) accumulation in culture supernatants of rhizosphere bacterial strains was quantified by high-performance liquid chromatography with two selective detectors. Twelve of 14 rhizobacterial strains produced detectable levels of IAA in culture filtrates. Two strains, 7SR5 and 7SR13, produced large concentrations of IAA (5–10 $\mu g\ ml^{-1}$), reduced root elongation, and increased shoot:root ratios of sugar beet when applied as seed inoculants. A significant linear relationship was observed correlating IAA accumulation of rhizobacterial strains with decreased root elongation and increased shoot:root ratios of sugar beet seedlings. The influence of bacterial sources of IAA on root elongation was also investigated by inoculating sugar beet seeds with IAA-producing strains of *Pseudomonas syringae* pv. *savastanoi*. *P. s.* pv. *savastanoi* strains 2009 and 2009-6 caused significant decreases in root elongation and increases in shoot:root ratios, whereas two derivative strains, 2009-3 and 2009-561, deficient in IAA production did not.

Many strains of rhizosphere bacteria influence plant growth or development when applied as seed or root inoculants (2,13,14,18,26,27). Numerous mechanisms have been postulated to explain these effects, including the production of phytohormones such as auxin derivatives and gibberellinlike substances that modify the plant's own pool of growth regulators and subsequently stimulate plant growth (1,4,9,32). Indole-3-acetic acid (IAA) or auxinlike substances have been detected in culture supernatants of several rhizosphere bacterial strains (11,15,25), including those that stimulate plant growth (1,3,4,9,23,32). These identifications, however, were based on the detection by auxin bioassay or chromogenic reagent of a compound that cochromatographed with IAA. The implications of these findings therefore can be viewed only as suggestive (25) because of the lack of positive identification and quantification of IAA accumulated in culture supernatants. Further, there is a need to examine whether the production of IAA by microorganisms in culture supernatants can be related to changes in root growth patterns when the producer strains are colonizing root surfaces.

We report here the identification and quantification of IAA in culture supernatants of rhizosphere bacterial strains by high-performance liquid chromatography (HPLC) combined with two selective detectors. We also report the correlation between the production of IAA by different strains and their influences on plant growth. Preliminary aspects of this work have been reported previously (17).

MATERIALS AND METHODS

Bacterial strains. Rhizobacterial strains used in this study are described in Table 1, and strains of *Pseudomonas syringae* pv. *savastanoi* used are described in Table 2. *P. s.* pv. *savastanoi* strains 2009 and 2009-6 produce IAA in culture and strains 2009-3 and 2009-561 do not (5,6,22).

Culture conditions. Cultures were grown in King's B medium (KBM) (12) or in a minimal medium described by Smidt and Kosuge (22). When indicated, Bacto peptone (Difco Laboratories, Detroit, MI) was substituted for proteose peptone No. 3 (Difco Laboratories) in KBM (KBM-B) and minimal medium was

supplemented with L-tryptophan (14 mM). Broth cultures were incubated in total darkness with shaking at 25 C for 3 days. Immediately after the broth culture was removed from the incubator, sodium citrate was added to a final concentration of 500 $\mu g\ ml^{-1}$. Cells were separated from broth medium by centrifugation at 8,000 g for 10 min. All harvesting procedures were carried out in dim light with samples maintained in covered ice baths.

HPLC. Culture supernatants were diluted into an injection solution (pH 3.0) of sodium citrate (500 $\mu g\ ml^{-1}$), EDTA (10 mM), methanol (20%), and butylated hydroxytoluene (2 $\mu g\ ml^{-1}$) before analysis by HPLC. Base conjugates of IAA were hydrolyzed by adjusting culture filtrates to pH 11.0, incubating at 60 C for 60 min, then readjusting to pH 3.0 before dilution into the injection solution.

IAA analysis was done two ways. The first HPLC system used a reverse-phase C8 column (25 cm × 4.2 mm i.d.) and a mobile phase of 0.05 M phosphate buffer (pH 3.0) containing 18% methanol, v/v . This system was equipped with a BAS LC-22 controller and LC-23 column heater set at 50 C, a Waters Associates M6000A solvent delivery system (1.0 ml/min flow rate), and a Waters Associates WISP 710B autosampler set to inject 100-μl samples. The system was equipped with two separate selective detectors connected in series as described by Sweetser and Swartzfager (28) and M. Hein (1983, Ph.D. thesis, University of Minnesota, Minneapolis). The first detector, a BAS electrochemical LC-3, had a working potential of 0.85 on glassy carbon vs. Ag/AgCl. The second, a Kratoes FS950 fluorescence detector, operated with a medium pressure mercury lamp, a 280-nm interference filter, and an emission filter with transmittance at 320 nm. Ratios of peak heights from the fluorescence (F) and the electrochemical (EC) detectors were calculated for IAA standards. This ratio (F/EC) serves as an accurate quantitative parameter for identifying IAA (28; M. Hein, 1983, Ph.D. thesis, University of Minnesota, Minneapolis). For each sample, peak heights corresponding to the retention times of IAA were measured on chromatographs from each detector, and the F/EC ratio was calculated. The ratios from each of three replications of each culture supernatant were compared to an IAA standard using the Wilcoxson rank-sum test.

The second HPLC system used a Dupont ODS reversed-phase column of 7-nm particle diameter (4.6 mm × 25 cm) run at ambient temperature. The mobile phase was 25% tetrahydrofuran (Burdick and Jackson, Muskegon, MI 49442) adjusted to pH 3.0 with phosphoric acid. The Waters Associates M6000A solvent delivery system was used, and a Rheodyne 7125 injector was used to inject a

50-μl sample. The system was equipped with an Aminco filter fluorometer (emitting at 320 nm and exciting at 280 nm).

Thin-layer chromatography. Culture supernatants were adjusted to pH 3.0, extracted three times with an equal volume of ethyl acetate, evaporated to dryness in a flash evaporator, solubilized in a minimal volume of methanol, and spotted on Whatman LK6DF silica gel thin-layer chromatography (TLC) plates. Plates were developed in two separate solvent systems, CEF (chloroform:ethyl acetate:formic acid, 50:40:10) or EIA (ethyl acetate:isopropanol:ammonia, 45:35:20), as described by S. T. Liu (1977, Ph.D. thesis, University of California, Berkeley). Plates were sprayed with Ehrlich's reagent (0.2% *p*-dimethylaminobenzaldehyde in 1:1 mixture of 95% ethanol and concentrated HCl) immediately after removal from the developing chamber. IAA was detected by observing a blue band with an Rf corresponding to that of IAA standards. Recovery of IAA was determined by incorporating freshly prepared IAA standards in the culture medium before initiating the extraction procedure. The limit of detection of the IAA standard was 0.10 μg ml^{-1}.

Colorimetric analysis. Indole compounds react with Salkowski's reagent (0.01 M FeCl$_3$ in 35% HClO$_4$) to form a red chromophore with maximum absorbance at 530 nm (8). A color change was noted 30 min after addition of Salkowski's reagent to culture filtrates, and absorbance at 530 nm was recorded (Beckman model 35 spectrophotometer). The lower limit of indole detection was approximately 1 μg ml^{-1} by this method.

Sugar beet seed inoculation and growth. Bacterial cultures, grown on KBM plates at 28 C for 48 hr, were suspended in sterile water, washed by centrifugation, resuspended in sterile water, and adjusted to A$_{640}$ = 0.1. Sugar beet (*Beta vulgaris* convar. *crassas* (Alef.) J. Helm cultivar U13748) seeds were soaked for 10 min in a bacterial suspension or in sterile water before placement in sterile growth packs (Northrup-King Seed Co., Minneapolis, MN). Ten sugar beet seeds were placed in each growth pack, and four growth packs were prepared for each treatment. The growth packs were incubated in a laboratory at room temperature (20–23 C) in natural sunlight, with root zone maintained in darkness. The packs were watered initially with half-strength Hoagland's solution and later with tap water as needed. Seedlings were removed from the packs 10–12 days after planting, and germination, hypocotyl length, primary root length, branching pattern, and general root morphology were recorded.

Statistical analysis. Software provided by Statistical Analysis Systems (SAS, release 79.6, SAS Institute, Inc., Cary, NC) was used to analyze data. The SAS General Linear Models procedure was used to perform analysis of variance (F) and Student's t tests and to generate Fisher's least significant difference values. Wilcoxson rank sum tests were done by the SAS NPAR1WAY procedure.

RESULTS

IAA production by rhizobacteria. HPLC analysis of culture supernatants of 14 rhizobacterial strains (Table 1) indicated that 12 produced IAA in culture supernatants. Concentrations of accumulated IAA in culture supernatants of strain 7SR5 were comparable to those of *P. s.* pv. *savastanoi* 2009 (9.1 ± 0.02 μg ml^{-1}). Presence of IAA in these culture supernatants was confirmed on a second HPLC system and by comparing the F/EC ratio with an IAA standard. After hydrolysis, IAA concentrations in every case were indistinguishable statistically from those in the original culture supernatants, indicating that IAA was present in a free or a peptide conjugate form rather than an esterified form.

Comparison of indole and IAA production by rhizobacteria. Concentrations of indole (determined colorimetrically) and of IAA (determined by HPLC) in culture supernatants did not differ statistically in most cases. However, five strains (7SR13, MtCa7, 51627, I11, and 7SR1) produced an indole compound in addition to IAA, as indicated by a statistical difference between concentrations of total indole and free IAA. The production of other indole compounds by these five strains was confirmed by observation of Ehrlich's reagent reactive bands that did not correspond with IAA on TLC plates developed in two different solvent systems. IAA was constitutively produced by *P. s.* pv. *savastanoi* but not by any of the

TABLE 1. Rhizosphere bacterial strains, concentrations of IAA in culture supernatants, and effects of strains on sugar beet[w]

Strain	Taxonomic designation[x]	Relevant characteristic	IAA concentration (μg ml^{-1})	Sugar beet growth[y] Primary root length (mm)	Sugar beet growth[y] Shoot:root ratio
Beneficial					
B4	*Pseudomonas putida*	Increases yield of sugar beet (26)	0.21 ± 0.01[z]	82.7 abc	0.40 b
B10	*P. fluorescens-putida*	Increases yield of potato (14)	1.22 ± 0.02	74.5 abcd	0.44 b
A1	*P. putida*	Increases yield of potato and sugar beet (14,27)	0.44 ± 0.02	85.6 abc	0.39 b
E6	*P. fluorescens*	Increases yield of potato and sugar beet (14,17)	<d	88.3 ab	0.39 b
E8	*P. fluorescens-putida*	Increases yield of radish (13)	0.57 ± 0.03	82.5 abc	0.40 b
I11	Enterobacteriaceae	Increases yield of radish (13)	0.79 ± 0.01	75.5 abcd	0.46 b
CoDi17	Enterobacteriaceae	Stimulates seedling growth of corn (*unpublished*)	<d	86.9 abc	0.47 b
Deleterious					
7SR5	Enterobacteriaceae	Inhibits seedling growth of sugar beet (T. Suslow, *personal communication*)	9.25 ± 0.14	17.0 e	1.88 a
7SR13	Enterobacteriaceae	Inhibits seedling growth of sugar beet (26)	4.59 ± 0.03	21.5 e	1.71 a
Wasco 4	Enterobacteriaceae	Inhibits seedling growth of sugar beet (26)	7.53 ± 0.10	70.9 bcd	0.49 b
51627	Enterobacteriaceae	Inhibits seedling growth of lettuce (*unpublished*)	1.33 ± 0.02	94.8 a	0.39 b
7SR8	*P. fluorescens-putida*	Inhibits seedling growth of sugar beet (T. Suslow, *personal communication*)	0.45 ± 0.03	78.8 abcd	0.44 b
7SR1	*P. fluorescens-putida*	Inhibits seedling growth of sugar beet (26)	0.57 ± 0.01	67.3 cd	0.52 b
MtCa7	*Flavobacterium* sp. Bergey et al 1923	Inhibits seedling growth of sugar beet (26)	1.30 ± 0.01	60.5 cd	0.59 b
Water control				83.2 abc	0.40 b
LSD ($P = 0.05$)				20.9	0.31

[w] Cultures were incubated with vigorous shaking at 25 C in KBM-B. Cells were harvested at 72 hr and IAA quantified from peak heights of chromatograms from two HPLC systems.

[x] *Pseudomonas putida* Migula and *P. fluorescens* Migula were distinguished by standard tests (24). Strains with characteristics common to both species are designated *P. fluorescens-putida*.

[y] Sugar beet seeds were inoculated with a bacterial suspension before planting in sterile growth packs. Hypocotyl lengths and primary root lengths were recorded 10–12 days after planting and expressed as a shoot:root ratio. Values are the means of four experiments, each with four replications. Means followed by the same letter do not vary according to Fisher's least standard difference. A significant F value protects LSD values in each parameter.

[z] Concentration represents the mean ± standard error of the mean of these cultures. <d = IAA concentration of culture supernatants was below the detection limit of 0.10 μg ml^{-1}.

14 rhizobacterial strains tested. Indole accumulation by these strains was detected only when the minimal medium was supplemented with tryptophan.

Effect of bacterial seed inoculants on growth of sugar beet. A significant linear relationship was observed between accumulation of IAA in culture filtrates of a rhizobacterial strain and its influence on primary root length ($r = -0.74$, significant at $P = 0.01$) or on the shoot:root ratio ($r = 0.74$, significant at $P = 0.01$) of sugar beet seedlings (Table 1). Seed inoculation with two rhizobacterial strains (7SR5 and 7SR13) that produced large amounts of IAA in culture significantly decreased primary root length and significantly increased shoot:root ratios of sugar beet seedlings, compared with control plants (Table 1). Seed inoculation with rhizobacterial strains that elaborated large amounts of IAA in culture supernatants, with the exception of strain Wasco 4, resulted in decreased primary root length and increased shoot:root ratios, compared with strains that produced relatively low concentrations of IAA.

Root elongation of sugar beet was also influenced significantly by seed inoculation with IAA-producing strains of *P. s.* pv. *savastanoi* (Table 2). *P. s.* pv. *savastanoi* strains that produce IAA in culture (2009 and 2009-6) (5,6,22) caused a significant decrease in root elongation of sugar beet, whereas mutant strains deficient in IAA production (2009-3 and 2009-561) did not.

DISCUSSION

The production of IAA by rhizosphere bacteria in culture supernatants was confirmed by HPLC followed by quantification with two selective detectors. Inoculation of sugar beet seeds with bacteria that produced large concentrations of IAA in culture was associated with decreased root elongation and increased shoot:root ratios. This inoculation did not affect hypocotyl length. These results are consistent with reported descriptions of the influences of exogenous sources of IAA on root elongation (7,29,30). The concentrations of IAA in culture supernatants of these deleterious bacteria averaged seven times those of beneficial strains. Furthermore, a significant linear relationship correlated IAA accumulation with decreased root elongation. The validity of this correlation was further supported by the finding that seed inoculation with *P. s.* pv. *savastanoi* strains that produced large amounts of IAA in culture supernatants decreased root elongation of sugar beet. Inoculation of seed with mutant *P. s.* pv. *savastanoi* strains deficient in IAA production caused no observable effect. Strain Wasco 4, which produced a relatively high amount of IAA in culture but did not stunt roots in growth pack tests, was the one exception to the pattern. This exception was not surprising, since IAA production by the bacteria on roots was not directly determined and Wasco 4 may not have produced sufficient amounts in the rhizosphere to cause an effect. Also, other metabolites produced by Wasco 4 could have masked the effect of IAA.

Because root elongation is inversely proportional to exogenous IAA concentration above a threshold of 10^{-6}–10^{-9} M depending on the plant (21,29), bacterial sources of IAA exceeding this concentration could be detrimental. The amount of IAA produced in culture by many of our test strains exceeded these concentrations. Bacterial production in the rhizosphere of high concentrations of any one phytohormone, such as IAA, may disrupt the hormonal balance critical to growth and development of root tissues. It has been shown with epiphytic bacteria that exogenous IAA can be transported into plant tissue (16), ultimately elevating endogenous IAA concentrations. Indirect effects of bacterial sources of IAA on plant growth also must be considered. These effects may include changes in microbial interactions, susceptibility of root tissues to colonization by rhizosphere microflora, and host resistance mechanisms. In studies addressing the influence of bacterial IAA production on colonization of plant tissues, mutants of *P. s.* pv. *savastanoi* deficient in IAA production multiplied in oleander leaves at a rate comparable to that of wild-type strains (22) but were deficient in epiphytic colonization (L. Varvaro and G. Surico. 1984. [Abstr.] *Pseudomonas* Working Group, Sounion, Greece.). The influence of IAA elaboration by rhizobacteria on their ability to proliferate in the rhizosphere is unknown, however.

The contribution of bacterial sources of IAA to the IAA pool in the rhizosphere depends on several factors, including the rhizosphere population sizes of IAA-producing strains and the amount of IAA produced by individual bacterial cells. Because IAA production by the rhizobacterial strains we examined depended on the presence of tryptophan in the culture medium, the composition and quantity of root exudates would be expected to influence IAA production by these strains in the rhizosphere. Tryptophan and related compounds have been identified in root exudates (10,19,20,31), but their concentrations and stabilities in a soil environment are unknown. In another study, a cursory examination of the rhizosphere population sizes of these 14 bacterial strains revealed no significant differences (*unpublished*). We assume, therefore, that any differences the bacteria may have produced in concentrations of IAA were relative to the inherent properties of the strain rather than the extent of epiphytic growth on the roots.

This and other studies (26,27) provide more evidence that some rhizosphere bacteria may act as "minor pathogens" and affect plant growth deleteriously by rhizosphere colonization and/or production of metabolites including, but not restricted to, phytohormones such as IAA. Caution should be exercised, however, in constructing theoretical models describing the influence of bacterial sources of IAA on root growth, since an approach for directly examining the concentrations and effects of IAA in the rhizosphere has not been perfected.

TABLE 2. Influence of seed inoculation with strains of *Pseudomonas syringae* pv. *savastanoi* on root elongation of sugar beet

Strain	Phenotype	Origin[x]	Primary root length (mm)[y]
2009	Wild-type, IAA+[z]	Field isolate (21); pIAA1	39 b
2009-6	Iaa+	Acridine orange-cured derivative of 2009 (5); pIAA1	53 b
2009-3	Iaa−[z]	Spontaneous mutant of 2009 (21)	103 a
2009-561	Iaa−	Spontaneous mutant of 2009 with insertion of IS51 at iaaM locus of pIAA1 (6,21)	111 a

[x] All strains were provided by T. Kosuge. Genes for IAA production are on pIAA1 of strain 2009.
[y] Means followed by the same letter are not significantly different (LSD = 22, $P = 0.05$; observed treatment $F = 22.2$, $P > F = 0.0001$).
[z] IAA concentrations (μg ml^{-1}) were 9.10 ± 0.02 and below 0.10 for strains 2009 and 2009-3, respectively. Concentrations were not determined for strains 2009-6 and 2009-561.

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