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Analysis of Indole-3-Acetic Acid and Related Indoles in Culture Medium from *Azospirillum lipoferum* and *Azospirillum brasilense*

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Analysis of neutral and acidic ethyl acetate extracts from culture medium of *Azospirillum brasilense* 703Ebc by high-performance liquid chromatography (HPLC) and combined gas chromatography-mass spectrometry demonstrated the presence of indole-3-acetic acid (IAA), indole-3-ethanol, indole-3-methanol, and indole-3-lactic acid. IAA in media of 20 strains of *A. brasilense* and *Azospirillum lipoferum* was analyzed quantitatively by both the colorimetric Salkowski assay and HPLC-based isotopic dilution procedures. There was little correlation between the estimates obtained with the two procedures. For instance, the Salkowski assay suggested that the culture medium from *A. brasilense* 703Ebc contained 26.1 μg of IAA ml^{-1}, whereas HPLC revealed the presence of only 0.5 μg of IAA ml^{-1}. Equivalent estimates with *A. brasilense* 204Ed were 10.5 and 0.01 μg of IAA ml^{-1}, respectively. The data demonstrate that the Salkowski assay is not a reliable method for measuring the IAA content of *Azospirillum* culture medium and that estimates in excess of 10 μg of IAA ml^{-1} should be viewed with particular caution. Metabolism of [2′-^{14}C]IAA by *A. brasilense* 703Ebc yielded radiolabeled indole-3-methanol, whereas roots of maize (*Zea mays* L.) seedlings gave rise to [^{14}C]oxindole-3-acetic acid and an array of polar metabolites. Metabolism of [2′-^{14}C]IAA by maize roots inoculated with *A. brasilense* 703Ebc produced a metabolic profile characteristic of maize rather than *Azospirillum* species.

Azospirillum species have been found in association with root systems of several grasses, including some of the more economically important cereals such as maize (*Zea mays* L.) (2, 7, 14). It has been suggested that the bacteria have the capacity to fix nitrogen when associated with cereal roots and that in tropical regions this ability can improve plant growth (3, 19). There is, however, an increasing realization that in many instances *Azospirillum*-induced growth promotion probably cannot be explained in terms of nitrogen fixation. Investigations into alternative mechanisms have focused primarily on bacterial production of growth regulators such as indole-3-acetic acid (IAA; Fig. 1). There are reports indicating that some *Azospirillum* strains can produce more than 30 μg of IAA ml^{-1} in medium supplemented with tryptophan (5, 11, 13, 17, 20), markedly in excess of IAA production by *Rhizobium* strains cultured in similar concentrations of the amino acid (10, 21).

The purpose of this study was to investigate the secretion of IAA and related indoles by *Azospirillum brasilense* and to quantitatively analyze IAA in cultures of a number of strains of *Azospirillum lipoferum* and *A. brasilense* isolated from roots of maize and teosinte. In addition, the metabolism of [2′-^{14}]IAA was investigated in *A. brasilense*, in *Z. mays* roots, and in *Z. mays* roots inoculated with *A. brasilense*.

MATERIALS AND METHODS

Plant material. Two maize (*Z. mays* L.) hybrids, designated (L903 × L902) and (L937 × L910), and the teosinte varieties Central Plateau and *Zea diploperennis* were field grown in a dark-red latossolo soil at the Universidade Estadual de Campinas, Brazil, in plots that had not received nitrogen fertilizer for 12 years. All plots were fertilized with 20 kg of N, K, and P ha^{-1} before planting. When plants reached the flowering stage, the root systems were removed from the soil and, after being shaken to remove particulate matter, were washed first in tap water, then in distilled water, and finally with 25 mM phosphate buffer, pH 7.0.

Isolation of bacteria. Excised roots were exposed to a 1% solution of chloramine T for 30 min, after which sections (0.8 to 1.0 cm long) were placed in 12-ml flasks containing 5 ml of N-free semisolid medium (6). After 24 to 48 h of incubation, the typical *Azospirillum* pellicle developed near the surface of the semisolid medium. Nitrogenase activity was determined by gas chromatography analysis of ethylene produced after incubation of cultures with 10% (vol/vol) acetylene in air at 25°C for 1 h (6). The cultures were enriched for *Azospirillum* cells by loop transfer of pellicle material to fresh semisolid medium. Further subculturing of *Azospirillum* cells was done with an agar medium containing mineral N and 20 mg of yeast extract liter^{-1} (6). Typical *Azospirillum* colonies were then transferred to N-free semisolid malate medium and incubated for 24 to 48 h for pellicle formation. The colonies were purified further and classified as *A. brasilense* and *A. lipoferum* according to procedures described by Baldani and Döbereiner (2). The classified strains were named as shown in Table 2.

***Azospirillum* culture.** Bacteria were grown in 125-ml Erlenmeyer flasks containing 50 ml of malate medium supplemented with 5 mM ammonium chloride and 100 mg of DL-tryptophan liter^{-1}. Flasks were shaken for 18 to 20 h at 32°C, and cultures were harvested by centrifugation at 2,800 × *g* for 15 min before the analysis of IAA and related indoles in the supernatant.

The number of cells in culture was determined by dupli-

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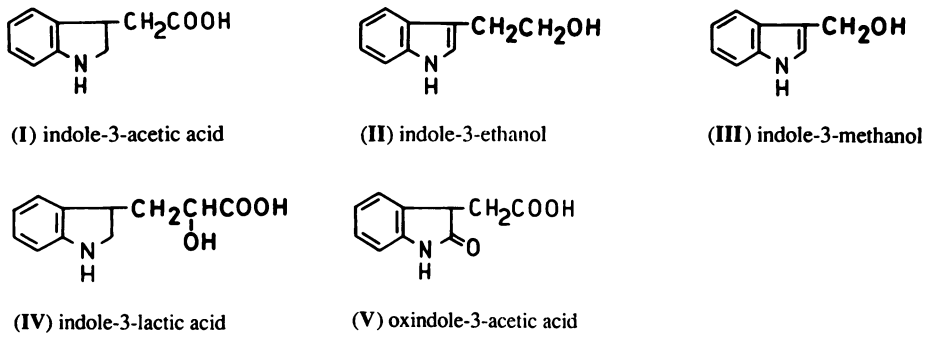

FIG. 1. Structures of IAA and related indoles.

cate plating of a medium dilution series on 1.5% agar containing 2.5 g of yeast extract, 5 g of tryptone, and 1 g of dextrose liter^{-1}. After incubation for 24 h at 32°C, the number of colonies formed was counted.

Qualitative analysis of IAA and related indoles. One liter of *A. brasilense* 703Ebc culture medium prepared as described above was centrifuged, and the antioxidant sodium diethyldithiocarbamate (5 mM) (9) was added to the supernatant, which was adjusted to pH 8.0 and slurried with insoluble polyvinylpyrrolidone (16). The filtrate was partitioned three times against 1/3 volumes of ethyl acetate, which were combined, treated with anhydrous sodium sulfate, and reduced to dryness in vacuo. The aqueous phase was adjusted to pH 3.0, and an acidic ethyl acetate fraction was obtained in a similar manner. Both the neutral and acidic ethyl acetate extracts were analyzed by gradient elution reversed-phase high-performance liquid chromatography (HPLC) with a fluorometric detector. Peaks of interest were further analyzed by combined gas chromatography-mass spectrometry.

Quantitative analysis of IAA. Two methods were used in parallel experiments with 20 strains of *A. lipoferum* and *A. brasilense*. The first involved colorimetric analysis of IAA in centrifuged culture medium with the Salkowski reagent (8, 11).

In the second method, an HPLC-based isotopic dilution procedure (16) was used. A [2′-^{14}C]IAA internal standard (specific activity, 63.9 mCi mmol^{-1}; 250 × 10^3 dpm; 311 ng) (Amersham International plc, Amersham, Buckinghamshire, United Kingdom) and antioxidant were added to 50 ml of centrifuged culture medium, which was adjusted to pH 8.0 and slurried with polyvinylpyrrolidone. After filtration, the aqueous phase was partitioned three times against 1/3 volumes of ethyl acetate, adjusted to pH 3.0, and partitioned similarly against diethyl ether. The acidic diethyl ether extracts were combined, dried, and purified by isocratic normal-phase HPLC with a radioactivity monitor operating in the heterogeneous mode. The [^{14}C]IAA peak was collected and dried, and a portion was analyzed by isocratic reversed-phase HPLC with a fluorescence detector (4). The fluorescent IAA peak in each sample was quantified by reference of the peak area to a standard curve. Radioactivity associated with the IAA peak was determined by liquid scintillation counting, using Ecoscint scintillation cocktail (National Diagnostics, Somerville, N.J.). This procedure enabled the amount of IAA in the centrifuged culture medium (Y) to be calculated from the isotopic dilution equation, $Y = ([C_i/C_f]-1)X$, of Rittenberg and Foster (15), where X is the amount of internal standard added to the sample, C_i is the specific activity of the internal standard, and C_f is the specific activity of the internal standard after dilution with endogenous IAA.

Metabolism of [2′-^{14}C]IAA. In metabolism studies with *A. brasilense* 703Ebc, [2′-^{14}C]IAA (4 × 10^6 dpm) was added to a 50-ml culture 16 h after inoculation. After a 4-h incubation period, the cells were centrifuged and antioxidant was added to the supernatant, which was then slurried with polyvinylpyrrolidone at pH 8.0 before partitioning to obtain neutral and acidic ethyl acetate extracts. Both extracts were analyzed by gradient elution reversed-phase HPLC, using a radioactivity monitor in the homogeneous mode.

In metabolism studies with roots, seeds of the maize single hybrid (L902 × L903) were germinated on moist tissue paper at 25°C for 3 days, after which individual seedlings were grown in 1.6 liters of Hoagland solution at 27°C under a 14-h photoperiod, with light supplied by Gro-lux fluorescent tubes with a radiation flux of ca. 50 W m^{-2} at plant height. When placed in Hoagland solution, the roots of half of the plants were inoculated with *A. brasilense* 703Ebc by the addition of 5 ml of bacterial culture (ca. 10^7 cells ml^{-1}). The seedlings were harvested after a 7-day growth period, and excised roots were used in metabolism studies with [2′-^{14}C]IAA.

Excised roots from five maize seedlings grown in the presence of *A. brasilense* 703Ebc were placed in a 10-cm petri dish with 10 ml of distilled water containing 4 × 10^6 dpm of [2′-^{14}C]IAA and incubated in darkness at 25°C. A similar experiment was carried out with roots from seedlings that had not been inoculated with *Azospirillum* cells. After a 4-h incubation period, the roots and the aqueous medium were macerated in methanol containing 5 mM sodium diethyldithiocarbamate in a Waring blender. The brei was filtered, and the methanolic extract was reduced to dryness and analyzed by gradient elution reversed-phase HPLC with a radioactivity monitor operating in the homogeneous mode. Radiolabeled peaks of interest were collected and further analyzed isocratically by normal-phase HPLC.

HPLC. Solvents were delivered at a flow rate of 1 ml min^{-1} by a Spectra Physics (San Jose, Calif.) SP 8700 liquid chromatograph. Samples were introduced by a Rheodyne (Cotati, Calif.) 7125 injection valve with a 250-μl loop. A 5-μm ODS Hypersil column (inside diameter, 250 by 5 mm; Shandon Southern Products, Runcorn, Cheshire, United Kingdom) eluted with various ratios of methanol in 0.5% aqueous acetic acid was used for both gradient and isocratic ion suppression reversed-phase separations. Isocratic normal-phase HPLC analyses were performed with a 5-μm Spherisorb CN nitrile column (inner diameter, 250 by 5 mm; Phase Separation Ltd., Deeside, Clwyd, United Kingdom) and a mobile phase of various ratios of hexane and ethyl acetate in 0.5% acetic acid (see reference 16).

HPLC column effluent was monitored with a Perkin-Elmer Corp. (Norwalk, N.J.) LS-3 spectrophotofluorometer (exci-

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tation, 280 nm; emission, 350 nm) fitted with a 16-μl flow cell. When radiolabeled compounds were analyzed, column effluent was directed to a Reeve Analytical (Glasgow, United Kingdom) radioactivity monitor operating in either the heterogeneous or homogeneous mode (16).

Combined gas chromatography-mass spectrometry. HPLC-purified fractions were silylated in 100 μl of acetonitrile-trimethylsilyltrifluoroacetamide (1:1, vol/vol) at 70°C for 15 min, reduced to dryness, and dissolved in 30 μl of hexane before analysis. Mass spectrometric analyses of the trimethylsilyl derivatives were performed on a Hewlett-Packard Co. (Palo Alto, Calif.) HP 5890 gas chromatograph linked to a 5970B mass selective detector equipped with an HP 9000 computer. Samples were introduced in the splitless mode (2 min of splitless time) at 225°C onto a cross-linked methyl silicone capillary column (inner diameter, 25 m by 0.31 mm) with a 0.52-μm film thickness. The column temperature was initially held at 60°C for 3 min and then programmed by 30°C min^{-1} to 130°C, followed by a 7°C-min^{-1} gradient to 235°C. The interface temperature was maintained at 250°C.

RESULTS AND DISCUSSION

Qualitative analysis of indoles. The production of IAA and related indoles was investigated by using *A. brasilense* 703Ebc, since preliminary studies with this strain, in which centrifuged culture medium was analyzed with the Salkowski reagent, indicated that after a 20-h incubation the liquid medium contained in excess of 20 μg of IAA ml^{-1}.

Neutral and acidic ethyl acetate extracts, obtained from 1 liter of centrifuged culture medium, were examined by reversed-phase HPLC with a fluorometric detector, which offers superior limits of detection and much greater selectivity for the analysis of IAA than does an absorbance monitor (16). The traces obtained are illustrated in Fig. 2. The neutral extract contained three fluorescent peaks: an early-eluting unknown substance; the major component which cochromatographed with the IAA precursor indole-3-ethanol (IEt; for structure, see Fig. 1); and a more retained constituent which cochromatographed with the decarboxylated IAA catabolite indole-3-methanol (IM; see Fig. 1 and 2A). The main peak in the acidic ethyl acetate extract cochromatographed with IAA, whereas one of the minor constituents, eluting before IAA, cochromatographed with indole-3-lactic acid (ILA; see Fig. 1 and 2B). The retention times of the three additional retained fluorescent peaks in the acidic ethyl acetate extract did not correspond with those of any of the available standards.

The fluorescent HPLC peaks in the neutral and acidic ethyl acetate extracts were collected individually, dried, and silylated, and the trimethylsilyl derivatives were analyzed by capillary gas chromatography-mass spectrometry. The spectra obtained (summarized in Table 1) confirmed the HPLC-based identifications of IEt, IM, IAA, and ILA. Recognizable spectra were not obtained with the trimethylsilyl derivatives of other HPLC peaks.

Quantitative analysis of IAA. Although IAA was identified by gas chromatography-mass spectrometry as an endogenous constituent of *A. brasilense* 703Ebc, quantitative estimates based on the HPLC analysis, albeit without an internal standard, implied that the amount of IAA present was ca. 2 orders of magnitude less than was measured when the Salkowski reagent was used to measure the IAA content of centrifuged culture medium. We decided to investigate this apparent anomaly in detail, as there are several reports in the literature of studies in which, because of the ease with which large numbers of *Azospirillum* cultures can be processed, the Salkowski assay has been used routinely to screen for mutant IAA producers (1, 11, 17).

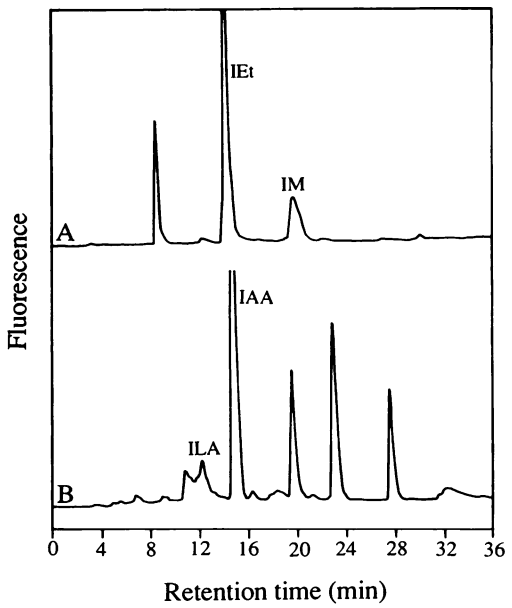

FIG. 2. Reversed-phase HPLC analysis of indoles in 1 liter of centrifuged culture medium from *A. brasilense* 703Ebc. (A) 1/1,000 portion of neutral ethyl acetate extract; (B) 1/1,000 portion of acidic ethyl acetate extract. Column, 5-μm ODS Hypersil (inner diameter, 250 by 5 mm); mobile phase, 30-min gradient, 30 to 70% methanol in 0.5% aqueous acetic acid; flow rate, 1.0 ml min^{-1}; detector, fluorometer (excitation at 280 nm and emission at 350 nm).

The IAA contents of culture media from 20 strains of *A. brasilense* and *A. lipoferum* were analyzed colorimetrically with the Salkowski reagent, and the data were compared with those obtained from parallel HPLC-based isotopic dilution analyses (Table 2). The HPLC analyses indicated that all of the *Azospirillum* cultures contained IAA. Three strains yielded <0.1 μg of IAA ml^{-1}, most in the range of 0.2 to 0.7 μg ml^{-1}, whereas five cultures contained >1.0 μg ml^{-1}, with the highest amount (4.5 μg ml^{-1}) detected in *A. brasilense* 901Ra. Estimates obtained with the colorimetric Salkowski assay ranged between 0.0 and 26.1 μg of IAA ml^{-1} and showed little correlation with the HPLC-based measurements. Strains seemingly containing >10 μg of IAA ml^{-1} when tested in the Salkowski assay were invariably found to contain much lower amounts when analyzed more rigorously by HPLC-based procedures. For instance, the

TABLE 1. Summary of electron impact negative-ion mass spectra obtained from trimethylsilylated HPLC fractions from neutral and acidic ethyl acetate extracts obtained from a 1-liter culture of *A. brasilense* 703Ebc

HPLC peak of:	Mass spectrum[a] (*m/z*)	Identity
IEt	[M+]305 (15), 290 (4), 216 (8), 202 (100)	IEt-TMS_2[b]
IM	[M+]291 (100), 276 (7), 202 (60)	IM-TMS_2
IAA	[M+]319 (40), 202 (100), 130 (12), 129 (9)	IAA-TMS_3
ILA	[M+]421 (5), 406 (4), 378 (3), 304 (3), 202 (100)	ILA-TMS_3

[a] Numbers in parentheses indicate percent intensity.
[b] TMS, Trimethylsilyl.

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TABLE 2. Comparison of estimates of the IAA contents of centrifuged culture media from 20 strains of *A. lipoferum* and *A. brasilense* obtained by the Salkowski assay and HPLC-based isotopic dilution analysis

Species and strain	IAA content of cells ($\mu g\ ml^{-1}$)		Content of medium (cells ml^{-1})
	Salkowski assay	HPLC	
A. lipoferum			
101Rd	3.5	0.4	4.5×10^8
404Rc	1.0	0.6	1.8×10^8
501Ra	6.0	0.05	2.6×10^8
502Rb	14.9	4.1	2.6×10^8
504Ea	1.0	1.4	5.7×10^8
701Rd	4.0	3.6	4.5×10^8
702Ra	0.0	0.2	7.3×10^8
702Rd	0.0	0.04	1.0×10^9
704Ed	7.0	0.7	9.3×10^8
902Rd	4.1	0.5	8.3×10^8
A. brasilense			
204Ed	10.5	0.01	4.0×10^8
303Ea	1.4	0.5	2.5×10^8
404Ed	0.0	0.2	2.5×10^8
601Rc	0.0	0.3	1.0×10^8
703Ebc	26.1	0.5	2.4×10^8
802Ra	4.3	0.5	2.3×10^8
803Ed	5.1	1.1	3.1×10^8
901Ra	15.0	4.5	4.4×10^8
903Rd	14.0	2.6	7.5×10^8
904Ed	8.5	0.7	6.9×10^8

colorimetric assay of the culture medium indicated that *A. brasilense* 703Ebc contained 26.1 μg of IAA ml^{-1}, whereas HPLC analysis revealed the presence of only 0.5 μg of IAA ml^{-1}. Similar analyses with *A. brasilense* 204Ed provided estimates of 10.5 and 0.01 μg of IAA ml^{-1}, respectively. These observations suggest strongly that inaccurate data are likely to be obtained when the Salkowski assay is used to analyze the IAA content of unpurified *Azospirillum* culture medium. In particular, estimates indicating the presence of >10 μg of IAA ml^{-1} should be viewed with caution.

High estimates of the IAA content of *Azospirillum* cultures have also been reported after analysis of acidic ethyl acetate extracts by HPLC with an absorbance monitor at 280 nm (5, 13). These figures too were likely inaccurate, since the lack of detector selectivity and the level of extract impurities make it probable that contaminants were mistaken for IAA (16).

Arguably, the most convincing report of IAA levels in *Azospirillum* cultures is that of Horemans and Vlassak (12). These investigators used a fluorometric detector and analyzed samples with both ion pair and ion suppression reversed phase HPLC. Estimates of ca. 40 ng of IAA ml^{-1} were obtained and confirmed by further analysis in the 2-methylindolo-α-pyrone assay (18). This figure is lower than those obtained with most strains in our investigation, which may be attributable to the use of different culture conditions and *Azospirillum* strains or the fact that Horemans and Vlassak (12) did not use an internal standard to account for sample losses. It is of interest that the quantitative estimates of the IAA content of *Azospirillum* culture medium obtained by HPLC-fluorescence procedures, both in our study and in the study by Horemans and Vlassak (12), are consistent with those reported to occur in *Rhizobium* cultures grown in media supplemented with similar amounts of tryptophan (10, 21).

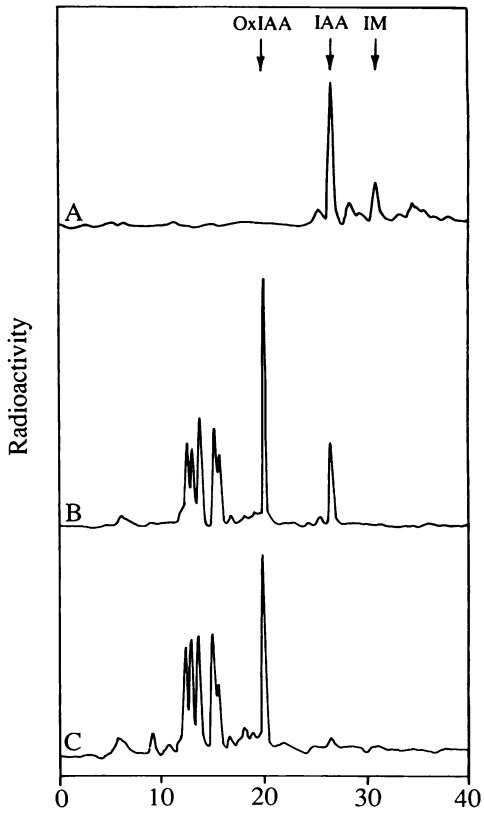

FIG. 3. Reversed-phase HPLC analysis of [2′-^{14}C]IAA metabolites produced by (A) *A. brasilense* 703Ebc, (B) maize roots, and (C) maize roots inoculated with *A. brasilense* 703Ebc. Column, 5-μm ODS Hypersil (inner diameter, 250 by 5 mm); mobile phase, 25-min gradient, 15 to 55% methanol in 0.5% aqueous acetic acid; flow rate, 1.0 ml min^{-1}; detector, radioactivity monitor operating in homogeneous mode. OxIAA, Oxindole-3-acetic acid.

Metabolism of [2′-^{14}C]IAA. The metabolism of [2′-^{14}C]IAA by *A. brasilense* 703Ebc, by maize roots, and by maize roots inoculated with *A. brasilense* 703Ebc was investigated. Extracts obtained after a 4-h metabolism period were analyzed by reversed-phase HPLC with a radioactivity monitor. The traces obtained are presented in Fig. 3. One of the radiolabeled metabolites of IAA produced by the *Azospirillum* cultures had the same retention time as did IM in reversed-phase HPLC. Metabolism of [2′-^{14}C]IAA by maize roots was much more complex, giving rise to a number of polar products, one of which had the same reversed- (Fig. 3B) and normal-phase HPLC retention properties as did oxindole-3-acetic acid (see Fig. 1). A similar metabolic profile was obtained with maize roots that had been inoculated with *A. brasilense* 703Ebc (Fig. 3C), which indicated that maize was making the predominant contribution to IAA catabolism in the inoculated roots. The data, however, give no indication as to whether the main source of IAA in the roots was bacterial or higher plant in origin.

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LITERATURE CITED

1. **Abdel-Salam, M. S., and W. Klingmüller.** 1987. Transposon Tn5 mutagenesis in *Azospirillum lipoferum*: isolation of indole acetic acid mutants. Mol. Gen. Genet. **210**:165–170.
2. **Baldani, V. L. D., and J. Döbereiner.** 1979. Host plant specificity in the infection of cereals with *Azospirillum* spp. Soil. Biol. Biochem. **12**:433–439.
3. **Bulow, J. F. K., and J. Döbereiner.** 1975. Potential for nitrogen fixation in maize genotypes in Brasil. Proc. Natl. Acad. Sci. USA **72**:2389–2393.
4. **Crozier, A., K. Loferski, J. B. Zaerr, and R. O. Morris.** 1980. Analysis of picogram quantities of indole-3-acetic acid by high performance liquid chromatography-fluorescence procedures. Planta **150**:366–370.
5. **De Francesco, R., G. Zanetti, P. Barbieri, and E. Galli.** 1985. Auxin production by *Azospirillum brasilense* under different cultural conditions, p. 109–115. *In* W. Klingmüller (ed.), *Azospirillum* III: genetics, physiology, ecology. Springer-Verlag KG, Berlin.
6. **Döbereiner, J., and V. L. D. Baldini.** 1979. Selective infection of maize roots by streptomycin-resistant *Azospirillum lipoferum* and other bacteria. Can. J. Microbiol. **25**:1264–1269.
7. **Dommergues, Y., J. Balancheau, C. Rinaudo, and P. Weinhard.** 1973. Non-symbiotic nitrogen fixation in the rhizospheres of rice, maize and different tropical grasses. Soil Biol. Biochem. **5**: 83–89.
8. **Ehmann, A.** 1977. The Van Urk-Salkowski reagent—a sensitive and specific chromatogenic reagent for silica gel thin-layer chromatographic detection and identification of indole derivatives. J. Chromatogr. **132**:267–276.
9. **Ernstsen, A., G. Sandberg, and A. Crozier.** 1986. Effect of sodium diethyldithiocarbamate, solvent, temperature and plant extracts on the stability of indoles. Physiol. Plant. **68**:519–522.
10. **Ernstsen, A., G. Sandberg, A. Crozier, and C. T. Wheeler.** 1987. Endogenous indoles and the biosynthesis and metabolism in indole-3-acetic acid in cultures of *Rhizobium phaseoli*. Planta **171**:422–428.
11. **Hartmann, A., M. Singh, and W. Klingmüller.** 1983. Isolation and characterization of *Azospirillum* mutants excreting high amounts of indoleacetic acid. Can. J. Microbiol. **29**:916–923.
12. **Horemans, S., and K. Vlassak.** 1985. Production of indole-3-acetic acid by *Azospirillum brasilense*, p. 98–108. *In* W. Klingmüller (ed.), *Azospirillum* III: genetics, physiology, ecology. Springer-Verlag KG, Berlin.
13. **Jain, D. K., and D. G. Patriquin.** 1985. Characterization of a substance produced by *Azospirillum* which causes branching of wheat root hairs. Can. J. Microbiol. **31**:206–210.
14. **Neyra, C. A., and J. Döbereiner.** 1979. Nitrogen fixation in grasses. Adv. Agron. **29**:1–38.
15. **Rittenberg, D., and G. L. Foster.** 1940. A new procedure for quantitative analysis by isotopic dilution with application to the determination of amino acids and fatty acids. J. Biol. Chem. **133**:737–744.
16. **Sandberg, G., A. Crozier, and A. Ernstsen.** 1987. Indole-3-acetic acid and related compounds, p. 169–301. *In* L. Rivier and A. Crozier (ed.), Principles and practice of plant hormone analysis, vol. 2. Academic Press, Inc. (London), Ltd., London.
17. **Singh, M., and W. Klingmüller.** 1986. Transposon mutagenesis in *Azospirillum brasilense*: isolation of autrophic and Nif^- mutants and molecular cloning of mutagenized *nif*DNA. Mol. Gen. Genet. **202**:136–142.
18. **Stoessl, A., and M. A. Venis.** 1970. Determination of submicrogram levels of indole-3-acetic acid: a new, highly specific method. Anal. Biochem. **34**:485–488.
19. **Subba Rao, N. S.** 1981. Response of crops to *Azospirillum* inoculation, p. 137–140. *In* P. B. Vose and A. P. Ruschel (ed.), Indian international workshop on associative nitrogen fixation. CRC Press, Inc., Boca Raton, Fla.
20. **Tein, T. M., M. H. Gaskins, and D. H. Hubbell.** 1979. Plant growth substances produced by *Azospirillum brasilense* and their effect on the growth of pearl millet (*Pennisetum americanum* L.). Appl. Environ. Microbiol. **37**:1016–1024.
21. **Wang, T. L., E. A. Wood, and N. T. Brewin.** 1982. Growth regulators, *Rhizobium* and nodulation in peas. Indole-3-acetic acid from the culture medium of nodulating and nonnodulating strains of *R. leguminosarum*. Planta **155**:345–349.

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