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Mass Spectrometric Quantification of Indole-3-Acetic Acid in *Rhizobium* Culture Supernatants: Relation to Root Hair Curling and Nodule Initiation

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Indole-3-acetic acid (IAA) has been unequivocally identified in culture supernatants of *Rhizobium* strains by gas chromatography-mass spectrometry. A method for accurately quantitating IAA in bacterial culture supernatants, employing deuterium-labeled IAA as an internal standard, has been developed. Similar IAA concentrations were found in culture supernatants of chosen *Rhizobium* mutants (defective in nodule formation) and their corresponding parent strains. Since some of the mutants are known to adhere to root hairs, it can be concluded that root hair curling is not simply a consequence of IAA production by rhizobia.

There has been much speculation that indole-3-acetic acid (IAA) might play a role at various stages in the symbiotic relationship between *Rhizobium* bacteria and leguminous plants (see reviews by Dart [4] and Newcomb [11]). In particular, it has been postulated that IAA is the causal agent of root hair curling (7). IAA has been tentatively identified in culture supernatants of *Rhizobium* strains grown in the presence of tryptophan (8, 14) and in root growth medium of plants inoculated with *Rhizobium* (10). This identification was based on the detection, by auxin bioassay or chromogenic reagent (Ehrlich or Salkowski), of a compound that cochromatographed with IAA.

We report here the unequivocal identification, by the use of gas chromatography-mass spectrometry (GC-MS), of IAA in culture supernatants of different strains of *Rhizobium*. Furthermore, we have developed a rapid method for accurately quantitating the concentration of IAA in bacterial culture supernatants. These procedures were applied to culture supernatants of chosen mutant strains of *Rhizobium* defective in various stages in establishing symbiosis (Table 1). Our aim was to investigate whether any correlation exists between the ability of a mutant to produce IAA and the block in its ability to nodulate, which might provide some insight into the role played by IAA with respect to root hair curling and nodule initiation.

MATERIALS AND METHODS

Bacterial cultures. Bacterial strains were grown in the dark at 28°C in liquid Bergersen modified medium (BMM) (2), which contained 0.5 g of yeast extract per liter, or in liquid Trifolii modified medium (TMM), which was Trifolii medium (18) with the following additions per liter: 1 g of casein hydrolysate (enzymatic digest; GIBCO Diagnostics, Madison, Wis.), 1 g of sodium glutamate, 112 mg of uracil, 56 mg of L-phenylalanine, and 56 mg of L-tryptophan (final pH, 7.1). Cultures were continuously agitated at 200 rpm. The mutant and its corresponding parent strain were inoculated at the same density, and both grew at the same rates. The absorbance at 600 nm was regularly measured for these cultures throughout the experiments. Checks on strain purity and for the presence of contamination were made on all cultures. Cultures were harvested by centrifugation at 8,800 × *g* for 35 min at 4°C, and the supernatants were stored at −20°C for no more than 4 days (sufficient time to obtain the results of sterility checks) before analysis.

Quantitation of IAA. The culture supernatants were exposed only to dim light. To a known volume of culture supernatant, a known and appropriate quantity of deuterium-labeled IAA ([methylene-$^2H_2$]IAA; Stohler Isotope Chemicals, Azusa, California) was accurately added. A stock solution of [$^2H_2$]IAA (40 mg of ethanol per liter) was used for this purpose. The culture supernatant was adjusted to pH 8.0 and partitioned three times against an equal volume of redistilled dichloromethane. The dichloromethane phase (neutral indole fraction) was dried over anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure at a temperature of less than 30°C. This fraction was stored at −20°C before bioassay (see below). The aqueous phase was adjusted to pH 3.0 and partitioned as described above. The dichloromethane phase (acidic indole fraction containing IAA) was dried and evaporated as described above, taken up in 0.4 ml of methanol, and transferred to a vial. The sample was dried under a stream of nitrogen and then thoroughly dried by evaporation of redistilled dichlor-

TABLE 1. Properties of chosen bacterial strains and concentration of IAA in culture supernatants[a]

Strain	Relevant characteristics	Origin of strain (reference)	IAA concn (mean ± SEM of three cultures) (μg liter^{-1})
Rhizobium trifolii		15	274 ± 21.5
SU843	Nodulates and fixes nitrogen (Nod$^+$ Fix$^+$) on white and subterranean clovers		
8002	Transposon Tn*5*-induced Nod$^-$ mutant of strain SU843; binds to root hairs but does not cause root hair curling (Hac$^-$ phenotype)	15	Table 2
ANU845	Nod$^-$ derivative of SU843; has lost a plasmid of 110 megadaltons after the method of Zurkowski and Lorkiewicz (23); has less root hair adhesion (Roa$^-$ phenotype)	15	204 ± 16.2
ANU845 (pBR1AN)	Strain ANU845 carrying the clover-nodulating plasmid pBR1AN; nodulates white and subterranean clovers	15	233 ± 34.4
T12	Nod$^+$ Fix$^+$ strain; contains two plasmids, one 230 megadaltons and one 107 megadaltons	23	162 ± 24.3
T12 Nod$^-$	Nod$^-$ mutant of strain T12 isolated after growth at elevated temperatures; has lost the 107-megadalton plasmid (1); binds to root hairs but does not cause root hair curling	22	272 ± 17.0
SU847	Nod$^-$ mutant of strain NA34; does not bind readily to root hairs (Roa$^-$); has normal exopolysaccharide composition (15)	17	255 ± 26.7
SU329	Nod$^+$ Fix$^+$ strain TA1	16	711 ± 232.1
3156	Transposon Tn*5*-induced nodule initiation mutant of strain SU329; has defective infection thread branching (Inb$^-$ phenotype)	Unpublished	481 ± 68.3
R. leguminosarum			
897	Nod$^+$ Fix$^+$ nodules on peas; *phe-1 trp-12 str-37*; a derivative of strain 300	9	291 ± 8.5
6015	Nod$^-$ derivative of strain 897; *rif-392 nod-6007*; has a deletion in a 205-megadalton plasmid; binds to root hairs but does not cause marked root hair curling (Hac$^-$)	3	197 ± 9.6

[a] Cultures were incubated in 100 ml of TMM. Cells were harvested in early stationary phase and IAA was quantitated. Binding assays were performed by direct counting with a fluorescence microscope and by radiolabeling the bacteria (Badenoch-Jones et al., unpublished data). Root hair curling assays were performed by examination of root hairs under the light microscope by the Fahraeus slide technique (6).

omethane. Trimethylsilyl (TMS) derivatives of the sample and of IAA were prepared by dissolving the sample in 15 μl of acetonitrile and 15 μl of bis-(trimethylsilyl)trifluoroacetamide and then heating at 70°C for 10 min. A sample of the resulting solution was then analyzed by GC-MS (GC column packing, OV 17 [3%] or OV 101; temperature, 155 to 200°C at 10°C min^{-1}). The electron impact mass spectra were taken with a DuPont 21-491B instrument (ionization voltage, 70 eV; source temperature, 250°C) interfaced with a VG 2025 data system. The ratio of endogenous IAA ([^{1}H]IAA) in the culture supernatant to the internal standard ([$^2H_2$]IAA) was calculated from the integrals of the ion currents at m/z 202 and 204 obtained from complete mass spectral scans (m/z 400 to 190) by a calibration curve constructed for this purpose. Identical results were obtained with the molecular ion pair m/z 319 and 321.

Bioassay. For bioassay of auxin activity, the wheat-coleoptile straight-growth test of Nitsch and Nitsch (12) was used with the following modifications: seeds of *Triticum aestivum*, cultivar Gabo, were surface sterilized for 45 s with sodium hypochlorite (concentration of available chlorine, 0.5% [wt/vol]), rinsed three times with distilled water, and then soaked in distilled water for 30 min. Seedlings were grown at 26°C, and 24 h before harvest they were exposed to red light for 30 min. The coleoptiles were harvested when they were 17 to 20 mm long. Sections 10 mm in length were cut, and the primary leaf was removed. These sections were floated on the solutions to be tested (samples and standards dissolved in 2 mM phosphate buffer [pH 6.0]) at 26°C for 24 h, and then their lengths were measured with a MOP-AM03 digitizing system (Kontron Elektronic GmbH, Eching, Federal Republic of Germany) after projection of their shadows with a photographic enlarger. Each sample was assayed on two separate occasions.

Culture of rhizobia in the presence of plant roots. Pea seeds were surface sterilized, germinated on soft agar, and grown in darkness for approximately 6 days (M. A. Djordjevic, W. Zurkowski, and B. G. Rolfe,

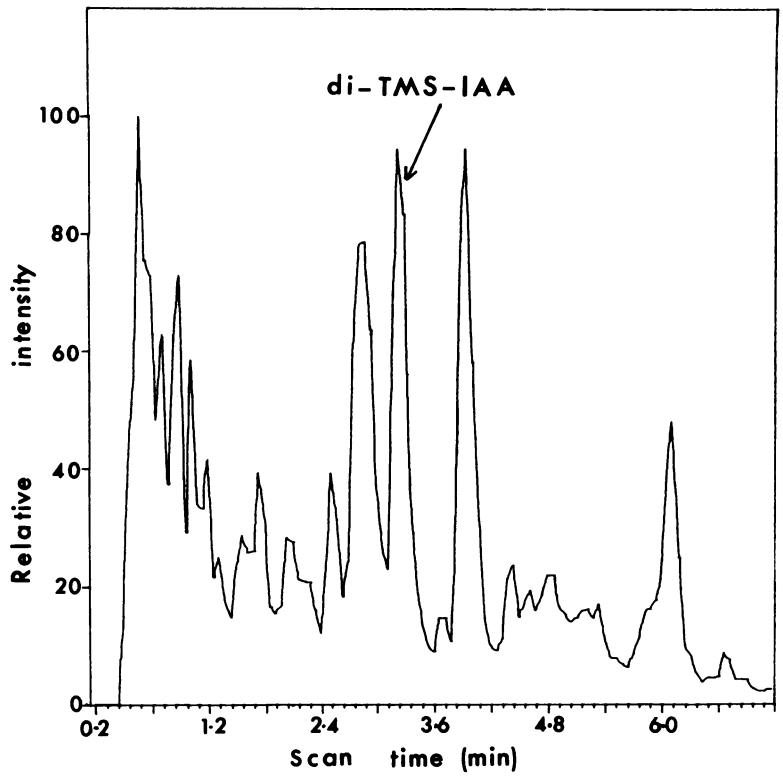

FIG. 1. Profile for the total ion current taken during GC-MS analysis of the acidic indole fraction derived from the culture supernatant of strain SU843 grown in TMM.

unpublished data). One seedling was then aseptically transferred to a flask containing 200 ml of liquid modified Fåhraeus medium (19), inoculated with either strain 897 or strain 6015, and then grown for 14 days under the following conditions: day, 16 h at 20°C, 100 μEinsteins m^{-2} s^{-1}; night, 8 h at 15°C. The medium was centrifuged (8,800 × *g* for 35 min at 4°C), and the supernatant was assayed for IAA (see above) after checks on strain purity and for the presence of contamination.

RESULTS AND DISCUSSION

When the acidic indole fraction of bacterial culture supernatants was reacted with bis-(trimethylsilyl)trifluoroacetamide with acetonitrile as solvent and the resulting mixture was subjected to GC-MS analysis, a peak was present which had a GC retention time (Fig. 1) and mass spectrum (Fig. 2) identical to those of authentic di-TMS-IAA. Detectable quantities of IAA were found in 500-ml samples of supernatants from 1-liter cultures of strain SU843 and the nonnodulating (Nod^-) mutant 8002 grown in different media containing various levels of added tryptophan (an IAA precursor). Only very small quantities of IAA were detected in uninoculated media incubated as described for the bacterial cultures: 1.1 and 1.6 μg of IAA $liter^{-1}$ for TMM and BMM, respectively. With the sample purification procedures described above, no $[^2H_2]$/$[^1H]$ back-exchange could be detected. The highest IAA concentrations were found in culture supernatants of media containing the highest concentrations of added tryptophan, a finding consistent with data obtained by Dullaart (5). However, there were no significant differences between the concentrations of IAA in the culture supernatants of these two strains whether they were grown in BMM or TMM (Table 2). Subsequent experiments were made with TMM, since the high concentration of IAA found in early stationary-phase cultures in this medium allowed accurate quantitations of IAA to be made on 100-ml batch cultures (Tables 1 and 2). Even after incubation for prolonged periods in TMM, similar concentrations of IAA were found in culture supernatants of strains SU843 and 8002.

The data presented in Tables 1 and 2 refer to the amount of IAA present at the time of harvest and not to the total amount produced by the bacteria, because IAA decomposes over the culture period. However, there is no evidence that the rate of decomposition of IAA (sum of biological and non-biological decomposition)

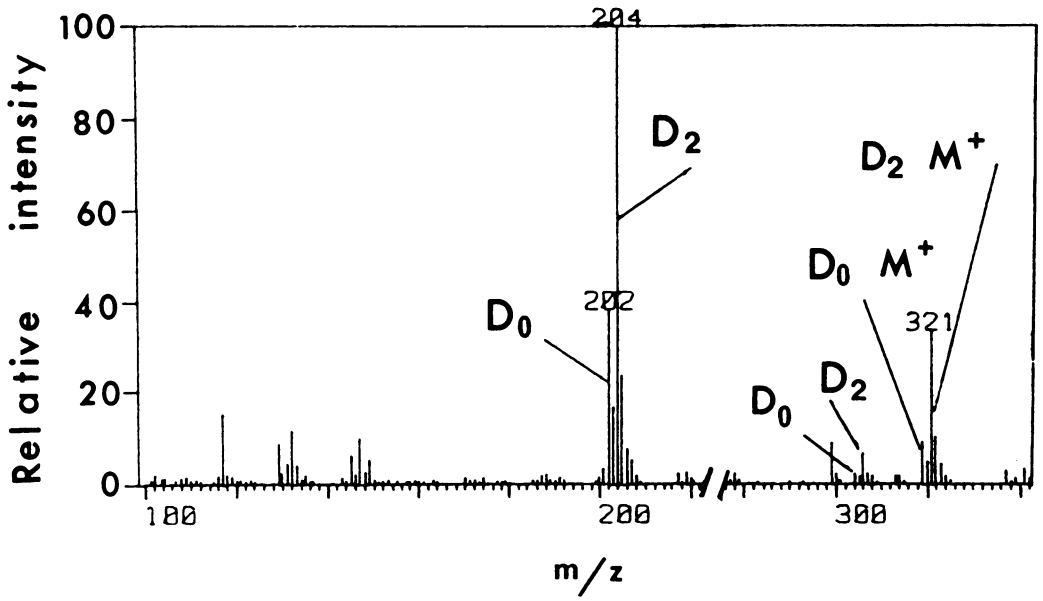

FIG. 2. Mass spectrum of the di-TMS-IAA peak shown in Fig. 1. The signals due to [$^2H_2$]IAA (D_2) and [^1H]IAA (D_0) are indicated. M^+ denotes the molecular ion. In the region deleted from the spectrum, there were no ions of relative intensity greater than 2.

differed for the two strains, SU843 and 8002. An assessment of this rate of breakdown of IAA was made by adding a known quantity of [$^2H_2$]IAA to the culture medium at the time of inoculation. Cultures were harvested at 20 h, and the culture supernatants were processed as described above, but without further addition of [$^2H_2$]IAA. Half of the sample of the acidic indole fraction was assayed by GC-MS. The remaining sample had an appropriate quantity of [^1H]IAA added and was then analyzed by GC-MS. From the ratios of [$^2H_2$]IAA to [^1H]IAA obtained for these two samples, it was possible to calculate the quantity of [$^2H_2$]IAA present at the time of harvest and therefore the percentage of breakdown of [$^2H_2$]IAA over the culture period. Duplicate values for the percentage of breakdown were as follows: SU843, 46 and 51%; 8002, 35 and 41%; medium alone, 66%. Since both synthesis and breakdown of IAA were occurring concurrently in the bacterial cultures, it was not possible, by the procedures described above, to distinguish between non-biological decomposition and that caused by the bacteria. It is clear, however, from the results of the control sample (medium alone) that non-biological decomposition was considerable. The higher figure for the percentage of breakdown of [$^2H_2$]IAA for the medium alone than for the bacterial cultures is almost certainly due to the larger pool of IAA in the cultures, derived from IAA production by the bacteria.

With the wheat-coleoptile straight-growth test, auxin activity of approximately equal magnitude was detected in the neutral indole fractions of culture supernatants of strains SU843 and 8002 grown in TMM (Table 3). However, no activity was detected in the control sample (TMM alone) or in culture supernatants of these strains grown in BMM. IAA concentrations of 10^{-3} M and higher inhibit coleoptile elongation in this bioassay, so the results obtained at the highest concentration of the neutral fraction are presumably due to supraoptimal concentrations of auxin. Tryptophol [2-(indol-3-yl)ethanol] is

TABLE 2. IAA concentrations of culture supernatants at the time of cell harvesting[a]

Culture conditions	IAA concn (μg liter^{-1})	
	Strain SU843	Strain 8002
Cells harvested in late log phase (mean ± SEM[b] of three 1-liter cultures)		
BMM culture medium	10 ± 3.5	20 ± 3.0
TMM culture medium	144 ± 16.0	143 ± 27.0
Cultures incubated in 100 ml of TMM (values for two cultures)		
20-h incubation	41, 63	49, 53
35-h incubation	222, 242	212, 320
65-h incubation	478, 1,167	532, 1,048

[a] Cultures were grown and IAA was quantitated as described in the text.

[b] SEM, Standard error of the mean.

TABLE 3. Auxin activity of neutral indole fractions derived from *Rhizobium* culture supernatants[a]

Medium	Strain	Final coleoptile length (% of buffer controls)[b]		
		50 ml[c]	5 ml[c]	0.5 ml[c]
TMM	SU843	84 ± 2.7[d]	112 ± 3.9[d]	112 ± 1.9[d]
TMM	8002	85 ± 2.9[d]	114 ± 4.5[d]	107 ± 2.7[d]
BMM	SU843	94 ± 2.3[d]	100 ± 1.1	101 ± 1.2
BMM	8002	102 ± 1.7	103 ± 1.3	99 ± 0.7
TMM	—	97	97	97

[a] The samples were the neutral indole fractions of the same culture supernatants for which IAA data are given in Table 2. The wheat-coleoptile straight-growth bioassay was performed as described in the text.

[b] Standards: 10^{-4} M IAA, 116 ± 3.0; 10^{-5} M IAA, 108 ± 2.5; 10^{-6} M IAA, 103 ± 2.0. Values are mean ± standard error of the mean.

[c] Equivalent volume of culture supernatant per milliliter of bioassay solution.

[d] Significantly different ($P < 0.05$) from 100.

possibly responsible for the auxin activity detected in the neutral indole fraction of TMM culture supernatants, since, at pH 8, it has a partition coefficient that favors dichloromethane (unpublished data) and has been shown to be active in the wheat-coleoptile bioassay (20). We are currently attempting to unequivocally identify indole compounds other than IAA in *Rhizobium* culture supernatants.

In addition to the above studies with strains SU843 and 8002, IAA determinations were made on the early stationary-phase culture supernatants of several other strains that were chosen because they provided examples of mutations or deletions of genetic information which result in defective nodule initiation and development (Table 1). There was no significant difference ($P > 0.05$) between the IAA concentrations of the early stationary-phase culture supernatants of the parent and the mutant for the following pairs of strains: SU843 and ANU845, SU843 and ANU845 (pBR1AN), SU329 and 3156, and SU329 and SU847. The mean IAA concentration of culture supernatants of strain 897 was significantly higher ($P < 0.01$) than that for 6015, and the value for strain T12 Nod$^-$ was significantly higher ($P < 0.05$) than that for T12. However, the physiological importance of these significant differences remains questionable for two reasons. First, all strains produced large quantities of IAA; second, in the case of the pair T12 and T12 Nod$^-$, it was the nonnodulating mutant that had the higher IAA concentration of its culture supernatant.

It is possible that a large difference in the production of IAA by the parent and mutant strains occurs in the presence of the plant. It has been shown, for example, that the presence of the plant is required for cytokinin synthesis by *R. phaseoli* strain 9-6 (13). Experiments were therefore carried out in which strains 897 and 6015 were grown in the presence of a pea plant. However, the amount of IAA detected in the pea root medium supernatants (pooled from three plants) 14 days after inoculation was similar for both strains (1.1 and 0.8 μg liter^{-1} for 897 and 6015, respectively). Controls yielded the following concentrations of IAA: uninoculated media in the presence of the plant, 0.2 μg liter^{-1}; strains 897 and 6015 maintained under identical conditions but in the absence of the plant, 0.4 and 0.7 μg liter^{-1}, respectively. Thus, the data from these experiments, in which rhizobia were cultured in the presence of plant roots, confirm the results of experiments with standard laboratory media.

In summary, for all strains tested, the values for IAA in culture supernatants were of the same order of magnitude. Thus, the genetic information coding for the enzymes required in the pathways of IAA biosynthesis does not reside on the large nodulation plasmid or nodulation genes, since mutants of *Rhizobium* which lack these genes or plasmid still retain the ability to produce IAA. Mutant strains 8002 and T12 Nod$^-$ do not cause marked or moderate root hair curling, and 6015 does not cause marked root hair curling, but these strains do adhere to root hairs and do produce IAA. Hence, we have established that moderate root hair curling in the course of the infection process is not simply a consequence of IAA production by rhizobia. Similarly, IAA alone is unlikely to be the factor responsible for marked root hair curling. Our data extend previous arguments (7, 21) that IAA alone is unlikely to be the causal agent of root hair curling. Our results obtained with mutant strain 3156 indicate that its defect in infection thread branching cannot be attributed to altered IAA production. Our data do not, however, preclude a role for IAA, either *Rhizobium* or plant derived, in other aspects of nodule development and maintenance, and we are further investigating the possible role of IAA.

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