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Communication

Identification of Gibberellins A_1, A_3, and Iso-A_3 in Cultures of *Azospirillum lipoferum*[1]

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ABSTRACT

Gibberellins A_1, A_3, and iso-A_3 were identified from aseptic cultures of *Azospirillum lipoferum* strain op 33 by capillary gas chromatography-mass spectrometry (GC-MS) and GC-MS-selected ion monitoring. There were 20 to 40 picograms (in GA_3 equivalents, estimated from bioassay) of gibberellins A_1 and A_3 per milliliter of cell culture (containing 10^9 cells).

The inoculation of several plant species (mainly grasses) with various strains of the 'free living' bacterium *Azospirillum* spp. often produces an enhancement of growth and/or yield, although these effects can be inconsistent, with quite variable responses in grain production (2, 9, 10, and references cited therein).

Among the possible mechanisms which have been proposed to explain the various effects of *Azospirillum* on higher plants is production of plant hormones by the bacterium (12). Most attention has been focused on auxin production (3, 4); however, GAs may also be involved since inoculation of wheat with *Azospirillum* produces an increase in ^{15}N uptake, and this effect can be mimicked by application of GA_3 to the wheat plant (7). Gibberellins have been identified from cultures of *Rhizobium phaseoli* (1).

While GA-like substances have been found in *Azospirillum* cultures (12), no physicochemical characterization has been reported.

In this paper we report the identification of GA_1, GA_3, and iso-GA_3, based on results from GC-MS and GC-SIM,[2] from aseptic cultures of *Azospirillum lipoferum* strain op 33.

MATERIALS AND METHODS

Azospirillum lipoferum, strain op 33 (an auxin overproducer [4]; Professor W. Klingmüller, University of Bayreuth, FRG), was aseptically cultured in 0.5 L of a liquid medium with NH_4Cl 2.5 g L^{-1}, malic acid 5 g L^{-1}, K_2HPO_4 0.5 g L^{-1}, $MgSO_4 \cdot 7H_2O$ 0.2 g L^{-1}, NaCl 0.1 g L^{-1}, FeEDTA 0.066 g L^{-1}, KOH 4.5 g L^{-1}, $CaCl_2$ 0.02 g L^{-1}, $NaMoO_4 \cdot 2H_2O$ 0.4 mg L^{-1}, $MnSO_4 \cdot H_2O$ 0.47 mg L^{-1}, BO_3H_3 0.56 mg L^{-1}, biotine/pyridoxal solution 1 mL L^{-1}. The bacteria were cultured for 48 h at 32°C (pH 6.5–6.8) with aeration, until the population reached approximately 10^9 cells mL^{-1}.

For extraction of GAs the culture was centrifuged for 15 min at 5000*g*, then the pellet was frozen with liquid N_2, ground and re-extracted with KH_2PO_4 buffer (pH 8.0), and again centrifuged. Both supernatants were pooled, acidified to pH 2.5, and partitioned five times with equal volumes of water-saturated EtOAc.

The acidic EtOAc phase was reduced *in vacuo* at 35°C, loaded onto 0.5 mm TLC plates (20 × 20 cm) of Silica Gel G (Sigma Chemical Co.), and developed for 15 cm with EtOAc:$CHCl_3$:AcOH (15:5:1). After elution with water-saturated EtOAc, 10 R_F zones were bioassayed in the dwarf rice cv. Tan-ginbozu microdrop bioassay (8) at 1/100 and 1/400 dilutions.

Eluates from R_Fs 0.3 to 0.4 showed the most bioactivity. These were submitted to reversed phase C_{18} (Waters Associates μ-Bondapak C_{18}, 300 × 3.9 mm i.d. column) HPLC, eluted at a flow rate of 2 mL min^{-1} with MeOH in 1% AcOH, with the following gradient: 10% MeOH for 10 min, 10 to 73% MeOH from 10 to 40 min, 73% MeOH from 40 to 50 min, and finally 100% MeOH from 50 to 60 min. Twenty fractions of 3 min (6 mL) were collected and again bioassayed in the dwarf rice microdrop assay (1/50 and 1/100 dilutions).

Table I. *Identification of GAs from A. lipoferum by GC-SIM and KRI*

Sample	KRI	Ions (m/z) with Relative Intensities[a]					
		506	491	448	447	377	376
GA_1	2674	(100)	(11)	(22)	(12)	(20)	(22)
Compound 1	2674	(100)	(11)	(16)	(8)	(17)	(18)
		504	489	445	370	347	297
GA_3	2698	(100)	(9)	(8)	(13)	(18)	(21)
Compound 2	2698	(100)	(7)	(7)	(14)	(13)	(15)
		504	489	475	445	369	311
Iso-GA_3	2641	(100)	(14)	(16)	(13)	(12)	(14)
Compound 3	2641	(100)	(10)	(18)	(13)	(15)	(11)

[a] Expressed as percent of parent ion (in parentheses).

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[2] Abbreviations: SIM, selected ion monitoring; AcOH, acetic acid; amu, atomic mass unit; EtOAc, ethyl acetate; KRI, Kovats' retention index; MeOH, methanol; MeTMSi, methyl ester trimethylsilyl ether.

Table II. *Major Ions from the Spectra of Authentic iso-GA_3 MeTMSi Standard and Compound 3 from Table 1*

Ion (m/z)	Compound 3	iso-GA_3 MeTMSi
	measured intensity × 10^{-2} (relative intensity)	*relative intensity*
73	720 (100)	(100)
75	178 (25)	(24)
91	28 (4)	(3)
141	34 (5)	(7)
155	22 (3)	(5)
157	35 (5)	(7)
167	31 (4)	(6)
180	8 (1)	(4)
181	15 (2)	(3)
191	14 (2)	(2)
193	33 (5)	(6)
194	20 (3)	(4)
207	29 (4)	(5)
221	31 (4)	(4)
223	23 (3)	(4)
238	45 (6)	(7)
239	31 (4)	(5)
265	14 (2)	(2)
267	19 (3)	(3)
271	10 (1)	(1)
281	21 (3)	(3)
309	17 (2)	(2)
311	25 (3)	(3)
355	23 (3)	(2)
369	39 (5)	(4)
370	28 (4)	(3)
371	26 (4)	(2)
387	15 (2)	(1)
431	20 (3)	(2)
445	36 (5)	(3)
459	18 (2)	(2)
475	47 (7)	(4)
489	25 (3)	(2)
504	250 (35)	(25)

Bioactivity was localized in two fractions, at the elution volumes expected of authentic [^{3}H] $GA_{1/3}$ and [^{3}H] GA_8.

These fraction residues were methylated with ethereal diazomethane and silylated with 1:1 pyridine:BSTFA (bis [trimethylsilyl] trifluoroacetamide) plus 1% trimethylchlorosilane (Pierce Chemical Co.), then dissolved in hexane and injected directly onto a Durabond DB-1-15N (15 m × 0.25 μm i.d.) capillary column (J and W Scientific Inc.) fitted in a Hewlett Packard 5790A Series GC with a capillary direct interface to a 5970B Mass Selective Detector. The GC temperature program was 60 to 195°C at 15°C min^{-1}, then 5°C min^{-1} to 275°C. Carrier gas (He) flow rate was 1 mL min^{-1}, the interface temperature was 280°C, and data aquisition was controlled by a HP 300 Series computer. For full spectra the mass range was 50 to 700 amu, scan rate 0.66 Hz; for SIM 6 ions for each compound were monitored, scan rate 1.34 Hz, dwell time 25 ms ion^{-1}. A mixture of *n*-alkanes was coinjected with the sample to allow later calculation of KRI (6).

RESULTS AND DISCUSSION

The evidence for identification of GAs from extracts of *Azospirillum lipoferum* strain op 33 is shown in Tables I and II. From the HPLC fraction of elution volume corresponding to [^{3}H] $GA_{1/3}$, GA_1, and GA_3 (compounds 1 and 2, respectively, from Table I) were identified, based on comparison of KRI and full spectra (of low intensity) with those of authentic standards. These identifications were confirmed by comparison of relative intensities of characteristic ions detected by SIM (Table I). From the HPLC fraction of elution volume corresponding to [^{3}H] GA_8, iso-GA_3 (compound 3 from Table I) was identified by a comparison of KRI and full spectrum (Table II) with that of the authentic compound.

Based on estimations by bioassay (of two dilutions of fractions after both TLC and HPLC), the *A. lipoferum* culture contained approximately 20 to 40 pg (GA_3 equivalents) mL^{-1} (i.e., per 10^9 cells) of GA_1 and GA_3. The amount of iso-GA_3 present was much greater than that of GA_1 and GA_3, based on the total ion current observed in GC-MS. Iso-GA_3 is about one-sixth to one-tenth as active as GA_3 on Tan-ginbozu, depending on the concentration applied (5). The question of whether iso-GA_3 is endogenous to the cell culture, or is made (as an artifact from GA_3) during extraction and purification cannot be answered. Iso-GA_3 is produced from GA_3 under basic conditions (11). However, cell culture conditions, and conditions during the extract work-up, were acidic (except during centrifugation, at which time the pH was 8.0). We would not expect appreciable isomerisation of GA_3 under these conditions.

It is always possible that samples might become contaminated with GA_3 during analysis. However, GA_3 is not used in large quantities in the laboratory in which the samples were analyzed. It is unlikely that samples would have been contaminated with GA_1 or iso-GA_3 at any time during cell culture or analysis.

The identification of bioactive GAs from pure cultures of *Azospirillum* provides a more complete basis for assessing the promotive effects of *Azospirillum* inoculation on growth and yield of higher plants.

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