Metabolism of 17,17[^{2}H$_2$]-Gibberellins A$_4$, A$_9$ and A$_{20}$ by *Azospirillum lipoferum* in Chemically-Defined Culture Medium

PATRICIA PICCOLI, OSCAR MASCIARELLI and RUBÉN BOTTINI[*]
Laboratorio de Fisiología Vegetal, Departamento de Ciencias Naturales,
Universidad Nacional de Río Cuarto, Campus Universitario,
5800 Río Cuarto, Argentina. Tel. +54-58-676103, Fax. +54-58-680280,
E-mail. rbottini@exa.unrc.edu.ar

Abstract

The metabolism of gibberellins A$_4$, A$_9$, and A$_{20}$ by *Azospirillum lipoferum* cultured in a chemically-defined medium was studied. *Azospirillum lipoferum* strain op 33 was cultured in i) NFb (Nitrogen Free biotine + 1.25 g/l NH$_4$Cl) medium + *A. lipoferum*, ii) NFb + *A. lipoferum* + 4 µg 17,17-[^{2}H$_2$]-GA$_4$ + 2.4 10^7 Bq [^{3}H]-GA$_4$, iii) NFb + *A. lipoferum* + 4 µg 17,17-[^{2}H$_2$]-GA$_9$ + 2.4 10^7 Bq [^{3}H]-GA$_9$, iv) NFb + *A. lipoferum* + 4 µg. 17,17-[^{2}H$_2$]-GA$_{20}$ + 2.4 10^7 Bq [^{3}H]-GA$_{20}$, at 80 rpm and 32°C for 6 days. After solvent partition and HPLC purification, metabolites were searched by radio-counting and their identity determined by capillary gas chromatography-mass spectrometry-selected ion monitoring. The different substrates did not interfere with either, the normal growth of the bacterium as assessed by OD, and viability determined by colony forming units/ml. Bacterial cultures metabolized all the substrates, converting 17,17-[^{2}H$_2$]-GA$_4$ to 17,17-[^{2}H$_2$]-GA$_1$, 17,17-[^{2}H$_2$]-GA$_3$, and 17,17-[^{2}H$_2$]-GA$_8$, while 17,17-[^{2}H$_2$]-GA$_9$ was metabolized to 17,17-[^{2}H$_2$]-GA$_3$. Also the conversion of 17,17-[^{2}H$_2$]-GA$_{20}$ to 17,17-[^{2}H$_2$]-GA$_1$ was confirmed. In order to find if some of the substrates are endogenous, the bacterium was cultured with different C/N ratios obtained by varying the NH$_4$Cl concentrations: 10, 7.5, 5, 2.5, 1.25, 0.625, 0.313, 0.08, and 0.02 g/l. Gibberellin

[*]The author to whom correspondence should be sent.

production was assessed from aliquots at 1, 3, 5, 7, 9, 11, and 13 days. Gibberellin A9 was characterized by capillary gas chromatography-selected ion monitoring from *Azospirillum lipoferum* cultures in early steps of the growth curve and in a medium with low C/N ratio. The results from this work in conjunction with others obtained by our group as well as those from the literature, support the hypothesis that GA1 and GA3 in *A. lipoferum* are produced following different pathways.

Keywords: *Azospirillum lipoferum*, metabolism, gibberellins

1. Introduction

Azospirillum spp. is a soil rhizobacterium with beneficial effects on plants, especially cereals (Patriquin et al., 1983; Michiels et al., 1989; Fulchieri and Frioni, 1994), and reports from literature suggest a great potential in promoting grain yield of crops (Summer, 1990; Okon and Labanderas-Gonzáles, 1994; Fulchieri and Frioni, 1994). Despite the fact that *Azospirillum* spp. shows the ability to fix dinitrogen (Baldani et al., 1983), the amounts of N_2 fixed represent only a small fraction of the plant requirements (Boddey and Döbereiner, 1988). Thus, increases in growth of Gramineae inoculated with *Azospirillum* spp. have been attributed, amongst other factors, to production of phytohormones (Tien et al., 1979; Okon, 1985; Okon and Kapulnik, 1986). These substances liberated by the bacteria would promote an increase of the radical system, including size and quantity of the root hairs with the concomitant enhancement of water and minerals uptake, which in turn would increase dry matter accumulation (Lin et al., 1983; Kapulnik and Okon, 1983; Jain and Patriquin, 1984).

Azospirillum spp. produces the gibberellins (GAs) GA_1 and GA_3 in chemically-defined culture medium, as it has been proven by capillary gas chromatography-mass spectrometry (GC-MS) (Bottini et al., 1989; Janzen et al., 1992). Inoculation with the bacterium increased *in vitro* root growth when inoculated to maize seedlings and altered the GA status in the organ (Fulchieri, 1992; Fulchieri et al., 1993). Although no other GA has been identified up today from *Azospirillum* spp. cultures, traces of GA_9 and GA_{19} have been found in former experiments relating the effects of different C/N ratios (Piccoli and Bottini, 1994a).

Gibberellin biosynthesis seems to come in a common pathway for both, the fungus *Gibberella fujikuroi* and higher plants, from mevalonic acid to GA_{12}-aldehyde (Crozier, 1982). The subsequent oxidation of the GA molecule at the C20 level may follow different pathways: a non-hydroxylative; an early 12α-hydroxylative; an early 13α-hydroxylative; or an early 3β-hydroxylative. The early 13α-hydroxylative pathway has been proposed as the one operating

in maize (Smith et al., 1991), *Pisum* (Reid and Ross, 1991) and *Cucurbita* (Graebe et al., 1991); e.g. GA_{12}-aldehyde => GA_{53} => GA_{44} => GA_{19} => GA_{20} => GA_1, or GA_{20} => GA_5 => GA_3. Although it has been frequently claimed specific to different plant species, recent results propose that those alternative pathways may operate in the same plant (Takahashi et al., 1986; Talon et al., 1990; Graebe et al., 1991; Rood and Hedden, 1994; Phillips et al., 1995). For instance, in *Brassica* spp. there is evidence that both, the non hydroxylative and the 13α-hydroxylative pathways are operative (Rood et al., 1987; Hedden et al., 1989). Additionally, it is also possible that in this species the early 3β-hydroxylative as well as the early 12α-hydroxylative pathways may be functional, since GA_{85} and GA_{89}, both representatives of these metabolic ways, have been characterized (Sheng et al., 1992 and 1993).

In *Gibberella fujikuroi* (Hedden et al., 1978; Crozier, 1982) GA_1 and GA_3 both come from 13α-hydroxylation of GA_4, with GA_7 as intermediate in the formation of the 1,2 double bond at ring A of the molecule for GA_3.

Results obtained with the fungus *Phaeosphaeria* sp. L487 (Kawaide et al., 1993) suggested the possibility of an alternative formation of GA_1, either by the 13α-hydroxylation of GA_9 to GA_{20} and then 3β-hydroxylation, or the inverse 3β-hydroxylation of GA_9 to GA_4 and then 13α-hydroxylation.

In previous results obtained with an experimental system similar to the one used in the present work (Piccoli and Bottini, 1994b), the bacterium was able to convert $17,17-[^2H_2]-GA_{20}$ to $17,17-[^2H_2]-GA_1$. Gibberellin A_{20} is inactive *per se* over shoot elongation (and perhaps root growth) in higher plants, and its transformation to GA_1 by a 3β-hydroxylase renders the structure active (Phinney, 1985). However, no other gibberellin has been identified apart from GA_1 and GA_3 from *Azospirillum* spp. cultures. The finding of iso-GA_1 (Bottini et al., 1989; Janzen et al., 1992) could be considered as an artifact of capillary GC (Gaskin and MacMillan, 1991) or of long exposures of GA_1 to high pH values (Takahashi et al., 1986).

This paper reports the conversion of $17,17-[^2H_2]-GA_4$ to $17,17-[^2H_2]-GA_1$, $17,17-[^2H_2]-GA_3$, and $17,17-[^2H_2]-GA_8$; of the $17,17-[^2H_2]-GA_9$ to $17,17-[^2H_2]-GA_3$; and of the $17,17-[^2H_2]-GA_{20}$ to $17,17-[^2H_2]-GA_1$ by *Azospirillum lipoferum* cultured in chemically-defined medium. Also the characterization of GA_9 by GC-SIM as produced by pure cultures of the bacterium is reported.

2. Material and Methods

Experiment 1

Azospirillum lipoferum strain op 33 (Piccoli and Bottini, 1994b) was grown in 500 ml flasks with 200 ml of NFb (Nitrogen Free biotine) medium as described

in Bottini et al. (1989) and Piccoli and Bottini (1994a and b), with malic acid (5 g/l) and NH_4Cl (1.25 g/l) as the sources for C and N, respectively. Seven sets of 3 flasks each contained:

1. NFb medium + *A. lipoferum*
2. NFb medium + *A. lipoferum* + 4 μg 17,17-$[^2H_2]$-GA_4 + 2.4 10^7 Bq $[^3H]$-GA_4 (high specific activity)
3. NFb medium + *A. lipoferum* + 4 μg 17,17-$[^2H_2]$-GA_9 + 2.4 10^7 Bq $[^3H]$- GA_9
4. NFb medium + *A. lipoferum* + 4 μg 17,17-$[^2H_2]$-GA_{20} + 2.4 10^7 Bq $[^3H]$-GA_{20}
5. NFb medium + 4 μg 17,17-$[^2H_2]$-GA_4 + 2.4 10^7 Bq $[^3H]$-GA_4
6. NFb medium + 4 μg 17,17-$[^2H_2]$-GA_9 + 2.4 10^7 dpm $[^3H]$-GA_9
7. NFb medium + 4 μg 17,17-$[^2H_2]$-GA_{20} + 2.4 10^7 dpm $[^3H]$-GA_{20}.

The 17,17-$[^2H_2]$-GAs were provided by L. Mander, Australian National University, and the $[^3H]$-GAs were a generous gift of R.P. Pharis, The University of Calgary, Canada.

The cultures were incubated 6 days in an orbital shaker water bath at 80 rpm and 32°C, and measurement of OD, pH, colony forming units/ml (CFU) and GA metabolites determination was done from 50 ml of microbial solution at 2, 4, and 6 days.

Experiment 2

Azospirillum lipoferum strain op 33 was cultured as per experiment 1, except that the different treatments were done by varying the NH_4Cl concentration as 10, 7.5, 5, 2.5, 1.25, 0.625, 0.313, 0.08, and 0.02 g/l. Thus, different C/N ratios were obtained ranging from 0.5 to 250. At 1, 3, 5, 7, 9, 11, and 13 days aliquots of 5 ml were taken from each culture and used to evaluate OD, pH, CFU, and GAs. In order to characterize and quantify GA production by the cultures, 20 ng of each of 17,17-$[^2H_2]$-GA_1, 17,17-$[^2H_2]$-GA_3, 17,17-$[^2H_2]$-GA_4, 17,17-$[^2H_2]$-GA_8, 17,17-$[^2H_2]$-GA_9, 17,17-$[^2H_2]$-GA_{19}, and 17,17-$[^2H_2]$-GA_{20} (L. Mander, The University of Adelaide, Australia) were added to the aliquots as internal standards.

Gibberellin purification

Bacterial cultures from experiments 1 and 2 were sonicated 10 min and centrifuged 10.000 xg 15 min. The supernatant was filtrated *in vacuo* through 0.22 μm cellulose filters and partitioned 4-fold with equal volume of ethyl acetate (saturated with 1% acetic acid) pH 2.8–3.0. The acidic ethyl acetate phase was dried, diluted with methanol 10% in 1% acetic acid, filtrated through 0.45 μm membranes and injected in HPLC reverse phase C_{18} column

(µBondapack, 300×3.9 mm, Waters Associates). The column was eluted 60 min with a 10/73% gradient of methanol in 1% acetic acid using a flow rate of 2 ml/min. Thirty fractions of 4 ml each were collected and dried.

Characterization of GA-metabolites

HPLC fractions from cultures of experiment 1 were re-dissolved in 150 µl of pure methanol, and radioactivity counted from an 50 µl aliquot. Those fractions showing radioactivity were grouped according their retention times, dried and derivatized to their methyl ester-trimethylsilyl ethers (MeTMSi) with ethereal diazomethane and BSTFA 1% TCMSi (Pierce Chem. Co.). After dissolving in 10 µl of hexane, 1 µl was injected on column in a capillary gas chromatography-mass spectrometry-selected ion monitoring system (GC-MS-SIM, Hewlett Packard 5890 Series II GC with a capillary direct interphase to a 5970B Mass Selective Detector). The GC column was a HP-1 (0.22 mm internal diameter and 25 m long, 0.1 µm film thickness) eluted with He (1 ml/min). The GC program was 60°C to 195°C at 20°C/min, then 4°C/min up to 260°C, then 10 min at 260°C. Three ions for each one of the $17,17-[^2H_2]$-GA, plus ion 85 for an hydrocarbon mixture were scanned in SIM mode at a rate of 0.8 cycles per second. A mixture of n-alkanes was co-injected in order to determine relative retention index according to Kovats (1958). Metabolite identification was made by comparison of the Kovats retention index (KRI) of the parent ions plus the 3 ions scanned, with those of authentic $17,17-[^2H_2]$-GAs.

Characterization of GAs produced by the cultures

For GA characterization from experiment 2, the HPLC fractions of the aliquots from cultures at different C/N ratios and different incubation times were bioassayed by the microdrop (0.5 µl) dwarf rice cv. Tan-ginbozu test (Murakami, 1968). Bioactive fractions were pooled according to their HPLC retention times and derivatized to MeTMSi as above. The GC-MS conditions were as described, and GA identification was assessed by coincidence of KRI and the comparison of 3 characteristic ions of the $17,17-[^2H_2]$-GA standard with the correspondent ions of the purported endogenous GA.

3. Results and Discussion

The different substrates did not affect the bacterial growth after 96 h of incubation. Both parameters, OD and CFU demonstrated that cultures had grown in the same way and they were well alive at the same time. Also pH

variations, which indicate malic acid consumption in the medium, were minimal amongst treatments (Table 1). In the case of NFB + the different substrates but without the bacterium, the pH remained unchanged (ca. 6.8, data not shown).

Table 1.   Culture growth measured as OD, bacterial viability measured as colony forming units (CFU)/ml, and pH variation along the time, of *Azospirillum lipoferum* strain op 33 cultures with [$^2H_2$]–gibberellins A4, A9 and A20. Data are the mean value of three replicates.

	2 Days pH/OD/CFU	4 Days pH/OD/CFU	6 Days pH/OD/CFU
Control	7.8/0.96/302	8.6/1.12/598	9.6/1.10/lay*
[$^2H_2$]-GA$_4$	7.5/0.94/288	9.0/1.30/512	9.5/1.30/303
[$^2H_2$]-GA$_9$	7.5/0.98/175	9.2/1.50/521	9.5/1.30/473
[$^2H_2$]-GA$_{20}$	7.9/1.10/287	9.2/1.20/534	9.4/1.20/386

*Opalescent layer covering almost all the agar surface after 96 h incubation at 32°C, which implies mucus formation throughout exopolysaccharide production by the bacterium.

Bacterial cultures metabolized all the substrates (Table 2), converting [^3H]-GA$_{4/9/20}$ to different metabolites with HPLC-Rt's similar to [^3H]-GA$_3$, [^3H]-GA$_1$ and [^3H]-GA$_{20}$. However, characterization of 17,17-[$^2H_2$]-GA$_1$, 17,17-[$^2H_2$]-GA$_3$, and 17,17-[$^2H_2$]-GA$_8$ as metabolites of 17,17-[$^2H_2$]-GA$_4$ by GC-MS (Table 3) was only possible; while 17,17-[$^2H_2$]-GA$_9$ was only metabolized to 17,17-[$^2H_2$]-GA$_3$. Also the conversion of 17,17-[$^2H_2$]-GA$_{20}$ to 17,17-[$^2H_2$]-GA$_1$ was confirmed. All of them correspond to precursors and metabolites of the different metabolic pathways known in fungi and higher plants (Takahashi et al., 1991).

We had previously demonstrated that *Azospirillum lipoferum* cultured in chemically-defined medium converts 17,17-[$^2H_2$]-GA$_{20}$ to 17,17-[$^2H_2$]-GA$_1$ (Piccoli and Bottini, 1994b), with no production of 17,17-[$^2H_2$]-GA$_3$. This fact was confirmed in the results presented here.

However, the *in vitro* conversion by the bacterium of one GA to another GA does not imply that this is a normal metabolic step under natural conditions, unless unequivocal characterization of the precursor as endogenous. In the case of *Azospirillum,* no other GA had been found up today apart from GA$_1$ and GA$_3$

Table 2. Metabolites expressed as % of radioactivity recovered from HPLC fractions and grouped according their HPLC–Rt as compared with those of authentic [^{3}H]–GA standards, in *Azospirillum lipoferum* strain op 33 cultures with 2.4 10^7 Bq of [^{3}H]-gibberellins A$_4$, A$_9$ and A$_{20}$, after 2, 4, and 6 days of incubation. Results are the mean value of three replicates.

Metabolite	Days of culture	Substrate [^{3}H]-GA$_4$	[^{3}H]-GA$_9$	[^{3}H]-GA$_{20}$
[^{3}H]-GA$_8$-like	2	8	–	9
	4	4	–	4
	6	3	–	3
[^{3}H]-GA$_{1/3}$-like	2	10	20	9
	4	45	17	12
	6	8	23	23
[^{3}H]-GA$_{20}$-like	2	44	30	69
	4	44	34	66
	6	86	70	62
[^{3}H]-GA$_{4/9}$-like	2	38	49	12
	4	5	45	16
	6	3	2	9

(iso-GA$_3$ has been reported as an artifact of capillary GC by Gaskin and MacMillan, 1991). In this sense, the identification of GA$_9$ as endogenous in *A. lipoferum* cultured in a C/N ratio of 1 and in an early phase of the growth curve (1 day incubation), reinforces the results of the metabolic studies. As can be seen from Table 4, both KRI and 4 characteristic ions of the purported GA$_9$ matched accordingly with those of authentic 17,17-[^{2}H$_2$]-GA$_9$ used as internal standard.

In maize, Smith et al. (1991) showed that GA$_1$ and GA$_3$ can commonly come via the early 13α-hydroxylation pathway from GA$_{20}$, with GA5 as intermediate in the case of GA$_3$. In *Gibberella fujikuroi* GA$_{20}$ is only a terminal product, without further conversion to any known GA, and GA$_1$ and GA$_3$ come from GA$_4$, via GA7 in the case of GA$_3$ (Crozier, 1982). However, in some other species GA$_{20}$ is converted to GA$_1$ (like in maize), while GA$_3$ comes from GA$_9$ => GA$_4$ => GA7 (Takahashi et al., 1986; Albone et al., 1990; Junttila et al., 1992; Rood and Hedden, 1994). In the fungus *Phaeosphaeria* sp. an alternative pathway has been suggested for GA$_1$ formation (Kawaide et al., 1993), but such

Table 3. Metabolites identified by GC-MS-SIM (according to KRI and relative intensities of 3 characteristic ions as compared with authentic 17,17-[^{2}H$_2$]-GAs standards) from *Azospirillum lipoferum* strain op 33 cultures with 17,17-[^{2}H$_2$]-GAs A$_4$, A$_9$ and A$_{20}$, after 4–6 days of incubation.

				Substrates							
[^{2}H$_2$]-GA$_4$				[^{2}H$_2$]-GA$_9$				[^{2}H$_2$]-GA$_{20}$			
KRI	508	493	450	KRI	508	493	450	KRI	508	493	450
[^{2}H$_2$]-A$_1$ std.											
2702	100	10	22	2702	100	10	22	2702	100	10	22
Metabolite											
2702	100	10	16	–	–	–	–	2702	100	10	22
KRI	506	491	447	KRI	506	491	447	KRI	506	491	447
[^{2}H$_2$]-A$_3$ std.											
2727	100	8	10	2727	100	8	10	2727	100	8	10
Metabolite											
2727	100	8	9	2727	100	8	10	–	–	–	–
KRI	596	450	381	KRI	596	450	381	KRI	596	450	381
[^{2}H$_2$]-A$_8$ std.											
2844	100	37	31	2844	100	37	31	2844	100	37	31
Metabolite											
2844	100	27	31	–	–	–	–	–	–	–	–

Table 4. GA$_9$ characterization by GC-SIM, based on similarities in KRI and relative intensities of 4 characteristic ions with authentic 17,17-[^{2}H$_2$]-GA$_9$, from 1 day-old *A. lipoferum* cultures with a C/N ratio of 1.

	KRI	332/330	300/298	272/270	245/243
[^{2}H$_2$]-GA$_9$ std	2371	12	100	70	55
GA$_9$	2371	8	100	47	38

a possibility has to be proven in further experiments with the utilization of labeled precursors. Having in mind that GA$_3$ is the unique metabolite of GA$_9$ in the present studies, and that the amounts of GA$_3$ normally found in *A. lipoferum* cultures are always superior to those of GA$_1$ (Bottini et al., 1989; Fulchieri, 1992; Janzen et al., 1992; Piccoli and Bottini, 1994a; Piccoli and

Bottini, 1995), the possibility of more than one alternative metabolic pathway is likely. Gibberellin A_1 and GA_3 could presumably come from different precursors. Gibberellin A_1 may be the result of a 3β-hydroxylation of GA_{20} as in the early 13α-hydroxylative pathway in maize (Phinney, 1985), while GA_3 may be the product of a non hydroxylative pathway with GA_9 as precursor. This is also substained by the finding that GA_1 and GA_3 production in *A. lipoferum* cultures was differentially stimulated by blue light (Piccoli and Bottini, 1995).

Even though $17,17\text{-}[^2H_2]\text{-}GA_4$ was metabolized to $17,17\text{-}[^2H_2]\text{-}GA_1$ $17,17\text{-}[^2H_2]\text{-}GA_3$, and $17,17\text{-}[^2H_2]\text{-}GA_8$, it is unlikely that this occurs under natural conditions, for several reasons. First, because GA_4 has not been found as endogenous in *Azospirillum* spp. cultures; second, because $17,17\text{-}[^2H_2]\text{-}GA_9$ metabolism did not produce detectable quantities of either $17,17\text{-}[^2H_2]\text{-}GA_4$, $17,17\text{-}[^2H_2]\text{-}GA_1$ or $17,17\text{-}[^2H_2]\text{-}GA_8$.

From an evolutionary standpoint, the general implication is that GAs are mostly the same in species belonging to three different kingdoms (bacteria, fungi, and higher plants), even though their metabolic pathways seem to vary even among species.

Thus, the results from this work in conjunction with others found by our group (Piccoli and Bottini, 1994b) as well as those from the literature (Kawaide et al., 1993), suggest that in *A. lipoferum* GA_1 is the biosynthetic product of GA_{20}, while GA_3 is produced by the conversion of GA_9.

Acknowledgements

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