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Metabolism of 17,17-[$^2H_2$]gibberellin A_{20} to 17,17-[$^2H_2$]gibberellin A_1 by *Azospirillum lipoferum* cultures

Patricia Piccoli and Rubén Bottini

SUMMARY

It was proven by capillary gas chromatography-mass spectrometry-selected ion monitoring analysis that *Azospirillum lipoferum* strain Al op33 cultured in chemically defined medium metabolized 17,17-[$^2H_2$]GA_{20} to 17,17-[$^2H_2$]GA_1

Key words: *Azospirillum lipoferum*, gibberellins, metabolism

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RESUMEN

Se comprobó mediante análisis por cromatografía gaseosa capilar-espectrometría de masa-monitoreo selectivo de iones que cultivos en medio químicamente definido de *Azospirillum lipoferum* cepa Al op33 metabolizaron [^3H]-GA_{20} a 17,17-[$^2H_2$]GA_1.

Palabras clave: *Azospirillum lipoferum*, giberelinas, metabolismo

Patricia Piccoli and Rubén Bottini. Laboratorio de Fisiología Vegetal, Departamento de Ciencias Naturales, Universidad Nacional de Río Cuarto, 5800 Río Cuarto, Argentina.

INTRODUCTION

Azospirillum lipoferum Al op33 produces the gibberellins (GAs) GA_1, GA_3 and iso-GA_3 in chemically defined medium (Bottini *et al.*, 1989). The same GAs have been characterized under similar conditions in *A. brasilense* (Janzen *et al.*, 1992) Iso-GA_3 may be an artifact of the culture basic conditions (Takahashi *et al.*, 1986), since pH of the medium after several days tends to alkalinization (Piccoli and Bottini, unpublished). However, no other GA has been characterized from *Azospirillum* cultures, and nothing is known in relation to their biosynthetic pathway

In the fungi *Gibberella fujikuroi*, GA_3 and GA_1 come from GA_4 in a metabolic pathway known as early-3β-hydroxylation (Crozier *et al*, 1982). In higher plants both, a non-hydroxylative and an early 13α-hydroxylative pathways are known (Takahashi and

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Kobayashi, 1991). In maize GA_{20} is the immediate precursor for GA_1, and GA_3 via GA_5 (Smith *et al.*, 1991), although in *Phaseolus coccineus* GA_1 may also come from GA_4 (Crozier *et al.*, 1991). Recently, Kawaide *et al.* (1993) found that the fungus *Phaeosphaeria* sp. L487 metabolizes GA_9 to GA_1 either, via GA_4 or via GA_{20}.

In the present paper the conversion of 17,17-[$^2H_2$]GA_{20} to 17,17-[$^2H_2$]GA_1 is reported by *Azospirillum lipoferum* AI op33 cultured in chemically-defined medium.

MATERIAL AND METHODS

Azospirillum lipoferum strain AI op33 (Abdel Salam and Klingmüller, 1987, kindly provided by Professor W. Klingmüller, University of Bayreuth, Germany) was cultured in a 500 ml flask with 200 ml NFB medium as previously described (Bottini *et al.*, 1989), except that NH_4Cl was in a concentration of 1.25 g liter^{-1}. Preliminary experiment, had shown that this N concentration as optimal for GA_3 production. Culture flask with medium was placed in a water bath with orbital shaking (80 r.p.m.), at 32°C for 20 h until OD = 1.1 and pH = 7.15. At this moment, 5.9 kBq of [^{3}H]GA_{20} (Professor R. P. Pharis, University of Calgary, Canada) plus 4 µg of 17,17-[$^2H_2$]GA_{20} (Professor L. Mander, University of Adelaide, Australia) were added to the medium, and incubation was continued for another three days. A blank flask was also incubated as described, except that no *Azospirillum* inoculum had been added. Both cultures were then submitted to the procedure that follows. Culture was centrifuged at 10.000 xg for 15 min, and the supernatant filtered under vacuo with 0,22 µm cellulose filters. Filtrate was submitted to partition four times with equal volumen of ethyl acetate (saturated with aqueous 1 % acetic acid). The ethyl acetate phase was evaporated under vacuo, re-taken with 10 % methanol in aqueous 1 % acetic acid, filtered with 0,45 µm filters, and injected in HPLC. HPLC was performed with a Konik 500 system, and a C18 µ-Bondapack (Waters Associates, 3.9 x 300 mm) column eluted at 2 ml min^{-1} with the following gradient of methanol in aqueous acetic acid (1 %): 1 to 10 min 10 % methanol, 10 to 40 min from 10 to 73 % methanol, 40 to 50 min 73 % methanol, 50 to 60 min 100 % methanol. Five fractiones were collected: I, from 14 to 20 min, II, from 21 to 28 min, III, from 29 to 34 min, IV from 35 to 60 min. These fractions corresponded to the retention times of ^{3}H-GAs A_8 (I), $A_{1/3}$ (II), $A_{5/20}$ (III), and less polar GAs or precursor-like GAs coming out with the methanol washing from the HPLC column (IV). Each HPLC fraction was then evaporated under vacuo, re-dissolved in pure methanol, and the radioactivity measured from aliquots. After being taken to dryness, the samples were re-dissolved in 10-20 µl of methanol anhidrous, methylated with ethereal diazometane (50-100 µl), mixed well, and left 30 min at room temperature. After solvent evaporation under N_2 stream the extracts were dissolved in 10 µl of dry pyridine, 50-100 µl of BSTFA [N,O-bis (trimethylsilyl) trifluoroacetamide] plus 1 % TMCS (trimethyl-chlorosilane) were added and silylated during 30 min at 65-85°C. The solvent mixture was again evaporated under N_2 stream, the dry samples dissolved in 6 µl of hexane and 1 µl was injected to the capillary column Durabond DB-1 15N (J & W Scientific Inc, cross-linked methyl silicone, 0.25 mm internal diameter, 0.33 µm film thickness, and 15 m long) fitted in the chromatographic system Hewlett Packard Series II GC-5970, coupled by a direct interphase to an HP-5898 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 interphase temperature was 280°C, and data adquisition was controlled by a HP 300 Series computer. Characteristic ions were monitored by a SIM mode, at a scan rate of 1.34 Hz, and dwell time 25 msec ion^{-1}

RESULTS AND DISCUSSION.

The percentage of radioactivity recovered in the different HPLC fractions is shown in Table I.

According these results, more than 50 % of the precursor was metabolized to more polar compounds, contained in the $GA_{1/3}$-like (II) and GA_8-like (I) fractions.

However, only [$^2H_2$]GA_1 from HPLC fraction II and [$^2H_2$]GA_{20} (the precursor) from fraction III were characterized from *Azospirillum* cultures incubated with [$^2H_2$]GA_{20}. The characteristic ions with their total and relative abundances for [$^2H_2$]-GA_1 are shown in Table II.

Table I. Total radioactivity recovered from the HPLC fractions.

	HPLC fraction			
	I GA_8	II $GA_{1/3}$	III GA_{20}	IV
Control	-	-	95	5
A lipoferum op 33 culture	17	35	45	3

Table II. Characteristic ions (m/z values, relative intensities [%] in parentheses) in the mass spectrum of the methylated GAs from fraction II (HPLC column), of the *A. lipoferum* strain op 33 culture compared with authentic [$^2H_2$]GA_1.

	Characteristic ions (m/z)							
	508	493	450	379	377	345	315	313
Fr.II compound	100	10	20	24	13	5	15	5
[$^2H_2$]GA_1	100	9	22	19	11	4	15	4

No other [$^2H_2$]GA metabolite was characterized. Although GA_3 is the major GA which *Azospirillum* produces (Bottini *et al.*, 1989; Janzen *et al.*, 1992), nor [$^2H_2$]GA_5 neither [$^2H_2$]GA_3 were found. Even-though the ability of the bacterium to perform the 3β-hydroxylation of [$^2H_2$]GA_{20} demonstrated in this work, the GA pathway does not seem to operate via the early 13α-hydroxylation as in maize (Smith *et al.*, 1991). The mechanism does also not resemble as found in *Gibberella fujikuroi* (Crozier, 1982), in which GA_{20} is a terminal GA with no conversion to 3β-hydroxylated GAs. The possibility of an alternative pathway as found *in Phaeosphaeria* (Kawaide *et al.*, 1993) has to be proven in further experiments through the utilization of [$^2H_2$]GA_4 and [$^2H_2$]GA_9 feedings.

Thus, *A. lipoferum* strain op 33 cultured in chemically defined medium converts 17,17-[$^2H_2$]GA_{20} to 17,17-[$^2H_2$]GA_1.

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