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Effects of C/N Ratio, N Content, pH, and Incubation Time on Growth and Gibberellin Production by *Azospirillum lipoferum*

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Abstract

Different C/N ratios did not substantially affect growth of *Azospirillum lipoferum* A1 op 33. However, under an initial concentration of NH_4Cl 1.25 g l^{-1} (C/N ratio 12.5) and pH 6.8, exopolysaccharide production was observed. This was correlated with a higher survival of the microorganism measured as colony forming units (CFU), and a substantial increase in gibberellin production (426 pg ml^{-1} of gibberellin A_3 accumulated after 5 d of culture), as measured by capillary gas chromatography-mass spectrometry-selected ion monitoring (GC-MS-SIM) with deuterated internal standards. The identification of gibberellin A_1 by selected ion monitoring, and of gibberellin A_3 by full spectrum was confirmed. The optimal production of gibberellin A_3 by pure cultures of *Azospirillum lipoferum* Al op 33 was obtained by accumulation after several days in the stationary phase, under conditions of high viability for the bacterium.

Keywords: *Azospirillum*, gibberellin

1. Introduction

Gibberellins (GAs) A_1, A_3 and iso-A_3 have been previously identified by physico-chemical methods in cultures of *Azospirillum lipoferum* strain Al op 33 (Bottini et al., 1989). The amount of GAs found after 48 hr of incubation was

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estimated with the dwarf rice cv. Tan-ginbozu microdrop assay (Murakami, 1968), as equivalent to 20–40 pg of GA_3 ml^{-1} of culture. No other GA has been characterized in chemically-defined culture medium of *Azospirillum* spp. (Bottini et al., 1989; Janzen et al., 1992).

GAs produced by the bacterium may be among the factors involved in the beneficial effects of *Azospirillum* spp. on seedling root growth in Gramineae. Actually, GA_3 had similar effects as *A. lipoferum* inoculation on promotion of root growth, especially in increasing hair density in areas physiologically active for nutrient uptake and water absorption (Fulchieri et al., 1993).

Janzen et al. (1992) have found that co-culture of *A. brasilense* with barley straw and the fungus *Trichoderma* (which degrades the straw increasing the C/N ratio) produced a substantial increase of GA. Candau et al. (1992) reported that GA synthesis in *Giberella fujikuroi* starts when N in culture medium is exhausted. This mechanism could be regulated by glutamine (Muñoz and Agosín, 1993).

The present work studies the effect of the C/N ratio, the total amount of N, the pH, and the incubation time, on growth and production of GAs by *A. lipoferum* A1 op 33.

2. Materials and Methods

Azospirillum *culture*

A. lipoferum strain Al op 33 (kindly provided by Prof. W. Klingmüller, University of Bayreuth, Germany) was grown in 500 ml flasks with 200 ml of NFB medium as previously described (Bottini et al., 1989). The C source was malic acid 5 g l^{-1}, and the N concentration varied as NH_4Cl 2.5, 1.25, 0.625, 0.312, 0.25, 0.15 and 0.025 g l^{-1}. Two-hundred μl of an *A. lipoferum* strain Al op 33 (OD: 1.4) suspension was added to each flask. In other experiments, a 1.25 g l^{-1} concentration was used, and the initial pH values (4.5, 6.8, and 9.5) adjusted by adding KOH. Each variant was done in triplicate. In all cases, the pH of the cultures varied as malic acid was consumed by the bacterium. After 2, 5 and 9 days of incubation at 32°C and continuous shaking (Lab-line orbit water bath, 80 rpm), OD, pH and colony forming units (CFU) were measured, and the presence of exopolysaccharides was determined from each triplicate. The cultures were then immediately processed for GA evaluation.

Colony forming units measurement and qualitative determination of exopolysaccharides

One ml dilutions (from 10^{-2} to 10^{-10}) of *Azospirillum* cultures were sown in Petri dishes on NFB medium plus agar, and after incubation for 96 hr at 32°C,

the CFU counted. For qualitative determination of exopolysaccharides, the procedure was done in nutrient agar plus calcofluor suspended in phosphate buffer pH 7.2 to a final concentration of 0.05%; after incubation for 72 hr at 32°C, colony fluorescence under 360 nm was checked (Del Gallo et al., 1989).

GA evaluation

For GA evaluation, cultures were disrupted by sonication, centrifuged at 10,000× g for 15 min, and the supernatant filtered in vacuo with 0.22 μm cellulose filters. After adding 20 ng of each one of the [$^2H_2$]-GAs A_1, A_3, A_4, A_8, and A_{20} as internal standards, filtrate pH was acidified to pH 3 and partitioned 4 times (100 ml each) with ethyl acetate (EtOAc) saturated with an aqueous solution of 1% acetic acid. The EtOAc phase (containing free GAs) was evaporated *in vacuo* in a rotary evaporator, and submitted twice to a short SiO_2 column (Koshioka et al., 1983), keeping in each case the soluble fraction in EtOAc/*n*-hexane. This fraction was evaporated and bioassayed by the dwarf rice cv. Tanginbozu immersion bioassay (Murakami, 1973). After dissolving with 10% aqueous methanol (MeOH), the EtOAc/*n*-hexane soluble fraction was filtered with a 0.45 μm filter for organic solvents and submitted to RP C_{18} μ-Bondapack (3.9×300 mm, Water Associates) HPLC column eluted with the following MeOH gradient in 1% acetic acid: 10% MeOH from 1 to 10 min, 10 to 73% MeOH from 10 to 40 min, 73% MeOH from 40 to 50 min, 100% MeOH from 50 to 60 min. Five fractions were collected for each HPLC injection: I, from 12 to 20 min; II, from 21 to 26 min, III, from 27 to 34 min; IV, from 35 to 46 min; V, from 47 to 60 min. These fractions correspond to the retention times of ^3H-GAs A_8 (I), $A_{1/3}$ (II), $A_{5/20}$ (III), $A_{4/9}$ (IV) and precursor-like GAs (kaurene, kaurenoic acid, V), respectively. Each HPLC fraction was evaporated under N_2 stream, and then derivatized to their methyl-esters by the following procedure: they were re-dissolved in 50–100 μl of MeOH anhydrous, 50–100 μl of ethereal diazomethane was added, and left for 30 min at room temperature. After solvent evaporation under N_2- stream, the fractions were dissolved in 10 μl of dry pyridine and 50–100 μl of BSTFA [N, O-bis (trimethylsilyl) trifluoroacetamide] plus 1% TMCS (trimethyl-chlorosilane) allowing 30 min at 65–85°C. After evaporation, fractions were dissolved in 6 μl of hexane, and 1 μl was injected onto a DB-1-15N (0.25 mm internal diameter and 15 m long) capillary column, in a gas chromatography system (GC) HP-5970, coupled by a direct interphase to an HP-5898 mass spectrometry detector (MSD). In each case, characteristic ions were monitored (selective ion monitoring-SIM), corresponding to internal standards and its respective non-deuterated equivalents. GA identification was made by comparison of the retention times of some of

these characteristic ions, and quantification by comparison of their relative intensities.

3. Results and Discussion

Table 1 shows growth (measured in OD) and viability (measured in CFU) of the bacterium related to the initial pH of the culture medium. The applied initial pH had a moderate effect on growth, but very significant influence on bacterium viability. When CFU was quantified in 10^{-8} dilution of the liquid culture grown on solid nutrient/agar medium, the presence of an opalescent layer was only observed at pH 6.8, as a result of exopolysaccharide production by the bacterium. This implies a great biomass production by the bacteria and exopolysaccharide production (Del Gallo et al., 1989). The presence of these types of substances excreted by the bacterium was confirmed by the characteristic fluorescence of the calcofluor test at 360 nm. However, with the other initial pHs tested, maximum viability was obtained after 5 days and was significantly lowered after 9 days. In the latter case, no exopolysaccharides were observed. These substances could be related to a higher survival of the microorganism, and thus in a better condition of producing secondary metabolites, such as GAs. Sadasivan and Neyra (1985) suggested that overproduction of exocellular polymers would induce floccular growth and concomitant transformation of vegetative cells to encysted forms, resistant to dessication under restrictive cultural conditions. Thus, the GAs produced by the bacterium could promote root growth with an increase in hair density, and lead to a better soil exploration by the plant root system. This effect might be particularly important in the early stages of seedling growth, especially in non-irrigated areas.

Cultures, with initial pH 6.8 and different NH_4Cl concentrations, reached

Table 1. Growth (measured as OD) and viability (measured as CFU in 10^{-8} dilution) of cultures of *Azospirillum lipoferum* grown in 1.25 g l^{-1} NH_4Cl at different initial pH

Initial pH	3 days OD/CFU	5 days OD/CFU	9 days OD/CFU
4.5	1.0/82	1.3/104	1.3/3
6.8	1.5/296	1.5/*lay*	1.5/*lay*
9.5	1.3/50	1.3/377	1.3/2

lay: opalescent layer covering almost all the agar surface after 96 hr incubation at 32°C, which implies mucus formation throughout exopolysaccharide production by the bacterium.

the stationary phase (measured by OD) after 3 d incubation at 32°C. Growth curves and the ability of the bacterium to form colonies under the diverse C/N ratios were not substantially different (Table 2). However, the best results

Table 2. Growth (measured as OD) and viability (measured as CFU in 10^{-8} dilution) of cultures of *Azospirillum lipoferum* grown at an initial pH of 6.8 and different concentrations of NH_4Cl

$[NH_4Cl]$ in g l^{-1}	3 days OD/CFU	5 days OD/CFU	9 days OD/CFU
2.5	1.2/296	1.5/*lay*	1.3/*lay*
1.25	1.0/369	1.3/*lay*	1.05/*lay*
0.625	0.87/185	1.25/265	1.1/105
0.312	0.76/109	1.1/164	0.9/96
0.25	0.7/99	1.1/104	0.95/66
0.15	0.72/92	1.12/162	0.9/143
0.025	0.5/66	0.9/102	0.85/96

lay: opalescent layer covering almost all the agar surface after 96 hr incubation at 32°C, which implies mucus formation throughout exopolysaccharide production by the bacterium.

Table 3. Quantification of GAs by GC-SIM in cultures (initial pH 6.8) of *A. lipoferum*. Results are expressed in pg ml^{-1}.

$[HN_4Cl]$ in g l^{-1}	Days of incubation 2	5	9
2.5	A_1 = 2.4	A_1 = nd	A_1 = nd
	A_3 = 0.9	A_3 = 0.9	A_3 = 0.9
1.25	A_1 = —	A_1 = —	A_1 = nd
	A_3 = —	A_3 = —	A_3 = 426
0.625	A_1 = —	A_1 = —	A_1 = nd
	A_3 = —	A_3 = —	A_3 = nd
0.312	A_1 = —	A_1 = —	A_1 = nd
	A_3 = —	A_3 = —	A_3 = nd
0.25	A_1 = 1	A_1 = nd	A_1 = nd
	A_3 = 1.1	A_3 = nd	A_3 = 1.6
0.15	A_1 = —	A_1 = —	A_1 = nd
	A_3 = —	A_3 = —	A_3 = nd
0.025	A_1 = nd	A_1 = nd	A_1 = nd
	A_3 = 1.1	A_3 = 0.6	A_3 = tr

nd, not detected; —, not run; tr, traces

were obtained with 1.25 g l^{-1} of NH_4Cl, both in biomass production (OD) and viability (CFU).

The production of GAs measured by GC-MS-SIM with deuterated standards was very low (or negligible) in all cases (Table 3), except for NH_4Cl 1.25 g l^{-1} concentration where 426 pg ml^{-1} of GA_3 were detected, approximately 10–200 times more than that obtained under the other conditions used in this and former experiments (Bottini et al., 1989). This effect could be given by N concentration and growth time, but not by C/N ratio, as no significant presence of GA_3 was accumulated after 2 days of growth in lower N concentrations. It is possible that the long incubation time at pH 9 or more created by malic acid consumption influences GA production by the bacterium. However, hormone production at high initial pHs was not promoted (Table 4). Thus, GA production appears to be correlated with culture viability, and polysaccharide excretion, favoured in turn by specific N concentration and initial pH.

GA_1 was identified by GC-SIM and GA_3 by GC-MS (full spectrum). The presence of GA_1 and GA_3, but not iso-GA_3 previously found (Bottini et al., 1989; Janzen et al., 1992), could be explained as a consequence of long incubation times at pH values over 9. This condition, to which the culture varies at the same as malic acid is consumed, would affect physicochemical conversion of GA_3 to iso-GA_3 (Takahashi et al., 1986).

Apart from GAs A_1 and A_3, which have been previously characterized (Bottini et al., 1989; Janzen et al., 1992) in *Azospirillum* spp., GAs A_9 and A_{19} were also tentatively identified. In fact, it was possible to detect the parental ions of these GAs at the retention times and in agreement with the parent ions of deuterated standards. However, other characteristic ions did not match well. These GAs are representatives of different biosynthetic pathways, the early 13α-hydroxylation (GA_{19}) and the non-hydroxylative one (GA_9; Takahashi and Kobayashi, 1991), which implies that GA_1 and GA_3 could originate from alternative pathways. This possibility is also supported by our results in

Table 4. Effect of initial pH on GA production in cultures of *A. lipoferum* after 9 d, estimated by the dwarf rice cv. Tan-ginbozu bioassay, from HPLC fractions I, II, III, and IV (see Material and Methods for details). Results are expressed in ng equivalents of GA_3.

Initial pH	GA_8	$GA_{1/3}$	$GA_{5/20}$	$GA_{4/9}$
4.5	ns	ns	ns	ns
6.8	0.1	25	0.25	0.2
9.5	ns	0.2	ns	ns

ns, not significant

which special culture conditions significantly promoted GA_3 production but not GA_1. Nevertheless, this is only a tentative identification and must be confirmed in further experiments.

Gibberellin A_3 identification was possible due to the relative high amount of the hormone in the culture medium. The relative abundance of characteristic ions of the substance produced by the bacterium agreed with those of the pure standard. The GA_3 quantification in extracts of the optimal culture medium by comparison of characteristic ions with those of a deuterated internal standard resulted in 426 pg ml^{-1} of endogenous hormone produced by *Azospirillum.*

In conclusion, optimal GA_3 production by pure cultures of *A. lipoferum* Al op 33 was obtained in cultures after several days in the stationary phase, and under high viability conditions for the bacterium.

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