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Production of Plant Growth Substances by *Azospirillum* sp. and Other Rhizosphere Bacteria

STEFAAN HOREMANS, KATLEEN DE KONINCK, J. NEURAY*, R. HERMANS** and KAREL VLASSAK

*K.U. Leuven, Laboratorium voor Bodemvruchtbaarheiden Bodembiologie, Kard Mercierlaan 92, B-3030 Leuven, Belgium *S.A. Carbochimique, Rue de Renory 21, B-4200 Seraing, Belgium*
***U.I.A., Dept. Biologie, Universiteitsplein 1, B-2610 Wilrijk, Belgium*

Abstract

The presence of zeatine, N^6-isopentenyladenine and N^6-isopentenyladenosine is shown in the culture supernatant of *Azospirillum brasilense* strain R07. No gibberellins could be detected. IAA was found in very large amounts in all *A. brasilense* strains tested but not in *A. lipoferum*. As compared to other rhizosphere bacteria neither the presence of these cytokinins, nor the absence of gibbereillins seems to be a very exclusive characteristic. The production of comparable amounts of IAA is rather exceptional.

Keywords: *Azospirillum* sp., zhirosphere bacteria, plant growth substances

Abbreviations: i^6-Ade: N^6-(Δ^2-isopentenyl) adenine; i^6-Ado: N^6-(Δ^2-isopentenyl) adenosine; IAA: indol-3-acetic acid; GA: gibberellin; Z: trans-zeatine; ZR: ribosyl-trans-zeatine; HPLC: high performance liquid chromatography; RIA: radio immuno assay; Ab: antibody; Nfb: nitrogen free broth; PGS: plant growth substances; Trp: tryptophane.

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1. Introduction

In addition to its capacity to fix gaseous N_2, *Azospirillum* spp. exhibit a number of phenotypic traits making them particularly suitable to survive in the rhizosphere (Balandreau, 1986). Following inoculation it promotes root hair development, causes alterations in the arrangement of cortex cells, increases mineral uptake and dry matter accumulation, improves the plant water status and enhances N_2 fixation (Okon and Kapulnik, 1986). In about half of the published field experiments some of the yield components were stimulated both under tropical and temperate conditions (Reynders and Vlassak, 1982). Often these effects could not be explained by N_2 fixation. Bacterial PGS production has been given as an alternative hypothesis. GA- and cytokinin like activity indeed have been found in the medium of *A. brasilense* (Tien et al., 1979). These observations were not confirmed up to now. IAA was released into the medium both in the presence (Reynders and Vlassak, 1979) and in the absence (Horemans and Vlassak, 1985) of added Trp. We investigated in some more detail the production of PGS by *Azospirillum* spp. and other rhizosphere bacteria.

2. Materials and Methods

Nfb medium (in g.dm^{-3} of distilled water): Malate: 5; KH_2PO_4: 0.6; K_2HPO_4: 0.2; $MgSO_4 \cdot 7H_2O$: 0.2; NaCl: 0.1; $CaCl_2 \cdot 2H_2O$: 0.025; $FeCl_3 \cdot 6H_2O$: 0.015; $Na_2MoO_4 \cdot 2H_2O$: 0.002; KOH: 4.9; biotine: 0.001; 4 ml of a bromothymolblue solution (50 g.dm^{-3} ethanol).

A. brasilense strain R07 was isolated from rice by Dr. Rinaudo. Local strains were isolated from the rhizosphere of maize or wheat by incubating rhizosphere samples in semi solid Nfb (agar 2 g.dm^{-3}, yeast extract 100 mg.dm^{-3}) for 4 days. A loopful from the upper 4 mm layer was transferred into fresh semi solid medium. Nitrogenase positive samples, exhibiting a typical subsurface pellicle were streaked on Nfb agarose (8 g.dm^{-3}) and incubated at 1% oxygen for 6 days. Single colonies were tested for nitrogenase activity. The procedure was repeated until purity. Isolates were comparable for at least 80 characteristics with type strains. Unidentified rhizosphere samples were isolated from the first enrichment culture in semi solid Nfb medium and further purified on nutrient agar. Per field site one strain was retained from the soil, 1 from the rhizoplane and 2 from surface sterilized crushed roots. Collections were made from grasslands, wheat-, barley- and maize-crops in late summer.

All analyses were done on the supernatant (20 min, 5000 g) of cultures grown under continuous shaking at 30°C for 6 days in Nfb containing 1 g.dm^{-3} NH_4Cl (no bromothymol blue). A 24 hr inoculum was washed twice with phosphate buffer (pH 6.6; 0.01 M) and 0.25 cm^3 was inoculated in 100 cm^3 erlenmeyer flasks containing 25 cm^3 of medium. The HPLC-fluorimetric analysis of IAA acid was done according to Horemans and Vlassak, 1985. The conversion of Trp to IAA was followed colorimetrically: 2 ml of 0.5 M $FeCl_3$ in 35% $HClO_3$ (1/50 v/v) was added to 1 ml of an appropriate dilution of supernatant. The O.D. was read at 530 nm after 20 min (room temperature). For a wide range of microorganisms a close relationship was found between the colorimetric assay and HPLC-on line U.V. photometry. Gibberellins were analysed using the microdrop dwarf rice internode elongation assay (Murakami, 1968). Cytokinins were analysed by ZR-AbRIA and/or i^6-Ado AbRIA according to Weiler (1980).

3. Results

Gibberellins

No GA activity could be detected (Table 1) in the supernatant of *A. brasilense*, strain R07. The stimulation found by the neutral ethylacetate fraction at 111 and 333 mm^3 of medium applied, although significantly differing from the no response value, cannot be considered as GA activity since this response is not increasing with increasing dose of medium applied. The absence of a response was not due to the presence of inhibitory substances since none of the supernatant fractions affected the response to 10 ng of GA_3 (Table 1).

We tested in the same way a mixture composed of the combined culture media of 72 unidentified rhizosphere bacteria and 30 out of them individually. The response was normally distributed around the equivalent of 30 pg per test (ranging from -8 to +120 pg per test), irrespective of the concentration applied. Therefore this stimulation, although significant at different occasions had to be considered as being aspecific and the presence of GA could not be shown.

Cytokinins

As analysed by RIA following Sephadex LH20 chromatography *A. brasilense* strain R07 excretes i^6-Ade, i^6-Ado and Z into the medium. No ZR was detected. Significant cross reactivity in the I^6-Ado-AbRIA (> 1 picomole.cm^{-3}) was found in 27 out of 41 rhizosphere isolates (data not

Table 1. Analysis of GA in different fractions of the culture medium of *A. brasilense* (grown 6 days at 30°C) using the microdrop dwarf rice internode elongation assay.

Amount of medium applied (mm^3)	Length (in mm) of the first and second internode in: The neutral ethyl acetate fraction	The acidic ethyl acetate fraction	The neutral butanol fraction
0	22.8±0.3	22.3±0.3	21.8±0.2
56	23.1±0.3	22.3±0.3	21.4±0.3
111	24.0±0.3*	22.0±0.2	21.8±0.3
333	23.7±0.3*	21.1±0.2	22.0±0.4
1000	22.3±0.3	22.0±0.3	21.8±0.3
0 + 10 ng GA_3	35.8±0.4*	37.3±0.5*	36.5±0.6*
333 + 10 ng GA_3	34.0±0.5*	37.0±0.3*	35.0±0.7*

* Significantly khigher than the controls ($p = 005$)

shown). For the same 41 bacteria the cross reactivity with ZR-ab was analysed. Two distinct populations, one at higher and one at lower activity could be recognized. Cross reactivity in the population showing the smallest response, although significant for about 1/3 of the strains was aspecific and/or due to variations in the assays since no increasing response was observed with increasing dose. For 7 out of 41 isolates (these showing a larger response) this response increased with increasing dose of medium applied and can be attributed to ZR or Z. This has been verified for 1 out of these isolates (the one showing the largest response). Following Sephadex LH20 chromatography cross reactivity was deteeted at the elution times of Z, i^6-Ade and i^6-Ado.

IAA

In the absence of Trp very large amounts of IAA were found in the culture medium of *A. brasilense* while *A. lipoferum* excreted only traces of IAA under the same conditions (Table 2). This difference is also obvious when the conversion is measured in the presence of $100\mu g.cm^{-3}$ Trp. Measuring the latter parameter on 200 rhizosphere isolates 16 out of them showed a high activity comparable to that of *A. brasilense*. The majority had a low activity comparable to that of *A. lipoferum*.

4. Discussion

No GA activity could be demonstrated in the culture supernatant of *A. brasilense* strain R07. This absence of any response was not due to inhibitory substances present in the medium. The discrepancy with the data

Table 2. Levels of IAA ($\mu g.cm^{-3}$) in the medium (6 days, 30°C), *A. brasilense* and *A. lipoferum* in the presence or absence of exogenously added Trp ($100\mu g.cm^{-3}$) measured by colorimetry and HPLC-fluorimetry

	Strain	IAA ($\mu g.cm^{-3}$)+ Trp	IAA ($\mu g.cm^{-3}$)-Trp
A. brasilense	R07	51	0.431
	A	55	0.456
	B	51	1.438
	C	49	0.664
	D	53	0.564
	E	57	0.367
	F	48	0.238
	G	49	0.778
	H	56	0.644
	I	54	0.595
A. lipoferum	J	11.5	–
	K	9	traces
	L	7.2	–
	M	3	traces
	N	2.75	–

of Tien et al. (1979) may be due to differences in the strain, the culture conditions or the bio-assay used. The absence of GA activity in the rhizosphere isolates is in good accordance with the low frequency of reports on GA activity in bacteria. i^6Ade and/or i^6-Ado were produced by *A. brasilense* R07 as well as by more than half of the rhizosphere isolates tested. In view of the long incubation periods (6 days) and since small amounts of the nucleosides were present, it is possible that these products are released from t-RNA and do not have a significant role in the rhizosphere interactions.

We confirmed the production of Z by *A. brasilense*. However 7 out of 41 rhizosphere isolates also showed a significant interaction with the ZR-ab RIA. At least for the one further checked this was due to the presence of Z. The production of cytokinins seems to occur at relatively high frequencies in the rhizosphere population and is thus not a unique property of *Azospirillum* spp. Before the production of cytokinins can be accepted as an explanation for observed inoculation effects, it should be demonstrated that the expression of this property is selectively enhanced *in situ*. As compared to other rhizosphere bacteria *A. brasilense* is converting very high amounts of Trp into IAA. *A. lipoferum* however, tested under the same conditions has a much lower activity, not differing at all from the majority of rhizosphere bacteria. This difference is paralleled by differences in their endogenous production.

As such *A. brasilense* produces IAA levels comparable to those found in the phytopathogenic strains of *Pseudomonas savastanoi* (Smidt and Kosuge, 1978).

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