Plant growth regulators and amino acids released by *Azospirillum* sp. in chemically defined media

D.S. Thuler[1], E.I.S. Floh[2], W. Handro[2] and H.R. Barbosa[1]

[1]Department of Microbiology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, and [2]Department of Botany, Institute of Biosciences, University of São Paulo, CP 11461, São Paulo 05422-970, Brasil

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ABSTRACT

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Aims: To investigate the ability of *Azospirillum* sp., a facultative endophitic diazotrophic bacterium, to release plant growth regulators (PGR) such as polyamines, ethylene, indoleacetic acid and amino acids in both combined-N and N-free cultures.

Methods and Results: The presence of those substances was analysed by HPLC. *Azospirillum* sp. is capable of releasing PGR and amino acids into the culture medium.

Conclusions: The type and quantity of the released substances varied, depending on the presence of combined-N in the medium.

Significance and Impact of the Study: A better knowledge of PGR produced by *Azospirillum* sp. has been gained.

Keywords: amino acids, *Azospirillum* sp., plant growth regulators.

INTRODUCTION

Azospirillum, a facultative endophytic N_2-fixing bacterium, occurring abundantly under tropical conditions, can positively influence plant growth, crop yields and N-content; these properties may be attributed to its ability to both fix nitrogen and synthesize plant growth regulators (PGR) such as auxins, gibberellins, cytokinins and ethylene (Tien *et al.* 1979; Strzelczyk *et al.* 1994). The improved plant growth following inoculation with *A. brasilense* may be attributed more to the production of PGR than to nitrogen fixation (Bashan and Holguin 1997).

Polyamines (PAs) are known to be ubiquitous in plant, animal and microbial cells (Davies 1995). PAs are able to regulate membrane functions and affect cell differentiation, interact with nucleic acids, etc.; physiologically, PAs have anti-senescence activity and ethylene is known as a senescence inducer (Galston and Kaur-Sawhney 1995). There is no information on the release of PAs in the genus *Azospirillum*.

Ethylene is a plant regulator, produced by higher plants as well as by micro-organisms. Ethylene plays several active roles in plants including germination, formation of root and shoot, seed germination and the response of plants to stress (Davies 1995). *Azospirillum* produces ethylene using methionine as a precursor (Strzelczyk *et al.* 1994).

Indole-3-acetic acid (IAA) influences plant processes, such as initiation of cell division, and promotes vascular differentiation (Gaspar *et al.* 1996). *Azospirillum* produces IAA, in concentrations that are typical of particular strain or species (Hartmann *et al.* 1983). Besides its hormonal functions, IAA is involved in the stimulation of ethylene synthesis (Glick 1995).

Amino acids can be excreted by free-living nitrogen-fixing organisms (Pati *et al.* 1994). Amino acids were not described in *Azospirillum* culture media with the exception of mutants of *A. brasilense* Sp Cd (Hartmann *et al.* 1983).

This paper compares the excretion of some PGRs (PAs, ethylene and IAA) and amino acids by *Azospirillum* sp. grown in N_2 or NH_4^+ as a nitrogen source.

Correspondence to: H.R. Barbosa, Department of Microbiology, Institute of Biomedical Sciences, University of São Paulo. Av. Prof. Lineu Prestes, 1374, São Paulo 05508-900, Brasil (e-mail: hrbarbos@icb.usp.br).

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MATERIALS AND METHODS

Micro-organism

To isolate the micro-organism, manioc roots (*Manihot* sp.) were washed in tap water, disinfected with 70% ethanol and 5% sodium hypocloride for 10 min and finally rinsed three times with sterile distilled water. The roots were macerated in sterile distilled water and the liquid phase was inoculated in semi-solid New Fabian broth (NFb) medium (Döbereiner 1980) and incubated for 48 h at 30°C. After the isolation procedure in NFb solid medium, a strain of an endophytic N_2-fixing bacterium was identified by molecular characterization. The acetylene reduction assay (ARA) test was employed to detect nitrogenase activity (Turner and Gibson 1980).

DNA extraction, PCR amplification and sequencing of 16S rDNA

Genomic DNA was obtained from bacterial cultures grown in Nutrient Broth (Difco) for 24 h, at 30°C, using the 'Wizard Genomic DNA Purification Kit' (Promega Cat.# A 1120; Promega, Madison, WI, USA), according to the manufacturer's instructions. PCR amplification of 16S rDNA was performed by using 30 ng of DNA in 25 μl reactions containing 2 mM $MgCl_2$, 20 μM each deoxynucleoside triphosphates, 0·3 M each bacterial universal primers 27f and 1525r (Stackebrandt and Goodfellow 1991) and 2 U *Taq* polymerase (Gibco BRL, Invitrogen Life Technologies, Calsbad, CA, USA) in 1× buffer. The reaction mixtures were incubated in a thermocycler MJ PTC-100 (MJ Research, Waltham, MA, USA) at 94°C for 2 min and then cycled 30 times: 94°C for 1 min, 55°C for 1 min and 72°C for 3 min, with final extension at 72°C for 10 min.

Purified PCR products were sequenced by using the 'Thermo sequence fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP' (Amersham Biosciences Corp., Piscataway, NJ, USA), following the manufacturer's instructions, and internal primers corresponding to conserved regions in the 16S rDNA, namely p1659 (5′-CTGCTGCCTCCCGTAGGAGT-3′; Lyamicheva *et al.* 1996), 782r (5′-ACCAGGGTATCTAATCCTGT-3′; Chun 1995), 530f (5′-CAGCAGCCGCGGTAATAC-3′; Lane 1991) and MG5f (5′-AAACTCAAAGGAATTGACGG-3′; Lyamicheva *et al.* 1996). Sequencing reactions were analysed in an ALF express (Amersham Pharmacia) sequencer.

Phylogenetic analysis of 16S rDNA sequence

Near complete 16S rDNA sequences were compared with sequences available at the Genbank (http://www.ncbi.nem.nih.gov) and RDP (Ribosomal Database Project, WI, USA, http://www.cme.msu.edu/RDP/html/index.html) databases. Sequences were analysed using the GDE software package (Genetic Data Environment, v. 2·2, gopher://megasun.dch.umontreal.ca:70/11/GDE). Distance matrices were calculated using DNADIST and the Jukes-Cantor model (Jukes and Cantor 1969), and phylogenetic trees constructed by using the neighbour-joining method (Saitou and Nei 1987), with bootstrap values calculated from 1000 replicate runs, using the software routines included in the PHYLIP ver. 3·5 package (Felsentein 1989).

Culture media

The NFb medium (Döbereiner 1980) was modified according to the substance to be analysed, as follows: to detect IAA, 0·5 g l^{-1} tryptophan (NFb_{Trp}) was added; to detect ethylene, 0·4 g l^{-1} methionine (NFb_{Met}) was added. IAA, ethylene, PAs and amino acids were researched either in the presence of 1·32 g l^{-1} ammonium sulphate (NFb_{AS}), or on its absence (NFb).

Microbiological and biochemical determinations. The micro-organism was grown in still cultures in a liquid NFb medium (Döbereiner 1980) at 30°C, for 72 h. About 40 ml of this culture were transferred to 1000 ml Erlenmeyer flasks containing 360 ml of the specific medium and incubated unshaken at 30°C. Fifteen millilitres of 250 h grown cultures were centrifuged (14 000 *g*, for 0·5 h) to detect PAs (Astarita *et al.* 2003b), IAA (Astarita *et al.* 2003a), and amino acids (Astarita *et al.* 2003c) by high-performance liquid chromatography (HPLC) in supernatants previously filtered through Millipore membranes (0·22 μm). The following PAs were analysed: putrescine (Put), spermidine (Spd), spermine (Spm), diaminopentane (Dap), cadaverine (Cad), hexylamine (Hex) and diaminoheptane (Dah).

To perform both growth and nitrogenase activity curves, aliquots from the NFb and NFb_{SA} cultures were taken, periodically, to determine: (a) colony forming units (CFU) counts (Barbosa *et al.* 1995), and (b) nitrogenase activity, by the C_2H_2 reduction method (Turner and Gibson 1980).

Ethylene assay

About 1 ml of the 72 h NFb cultures was transferred to 25 ml penicilin flasks containing 10 ml of a specific medium. The flasks were fitted with rubber plugs tightened with metal cowls and then incubated for 9 days at 30°C. In order to measure ethylene production, 1 ml of air samples was withdrawn from the flasks and analysed in a gas chromatograph (Shimadzu GC-14A, Shimadzu Corporation, Kyoto, Japan) with a PORAPAK-N 80/100 – INOX column operated isothermally at 70°C, with nitrogen as gas carrier and a flame ionization detector. Pure ethylene was used as a standard. Studies were performed in triplicate.

RESULTS AND DISCUSSION

By releasing plant hormone substances, soil microbes can stimulate plant growth and consequently enhance the production of plant metabolites and utilize them for their own growth (Gaudin *et al.* 1994). Hormones excreted by plant growth promoting bacteria produce physiological effects on certain species of plants (Glick 1995).

The isolated bacterium was identified as *Azospirillum* sp. Figure 1 shows that both NFb and NFb_{AS} grown cultures, when sampled, were in the stationary and decline phases, respectively. The absence of a death phase in NFb cultures suggests that the cells were actively liberating the analysed substances. In NFb_{AS} cultures, however, the substances might have been released by dead cells, as suggested by Zimmer *et al.* (1988). Nitrogenase activity declined during the stationary phase in the NFb culture and its inhibition was detected in the NFb_{AS} cultures.

Regarding PAs production, only Put, Spd and Spm could be detected (Fig. 2). The presence of NH_4^+ increased Put synthesis 14 times when compared with N_2. Spm and Spd were only present in NFb_{AS} cultures but in levels much lower than those found for Put under the same conditions. Species of free-living nitrogen-fixing bacteria were able to produce Put, Spm and also Cad (Goris *et al.* 1998).

It may be proposed that the regulation of intracellular PAs in *Azospirillum*, as in other bacteria, maintains an adequate concentration (Kashiwagi and Igarashi 1988). Inhibition of PA biosynthesis is accompanied by excretion when the intracellular level is high (Igarashi and Kashiwagi 2000). The detection of both Spm and Spd when the population is in subtle decline (Fig. 1) suggests that an active release of those substances is otherwise avoided, considering the Spm toxicity to bacteria (Tabor and Tabor 1985). The release of Put in both media may be seen as an interesting fact, once Bais *et al.* (1999) showed that the addition of Put to the culture medium affected plant growth and production of metabolites in hairy root cultures.

Ethylene excretion by *Azospirillum* sp. occurred in both NFb or NFb_{AS} cultures (Fig. 2). The presence of NH_4^+ enhanced production threefold, despite the lowest CFU concentration in this medium (Fig. 1). Strzelczyk *et al.* (1994) detected that the production of ethylene by this genus, in the presence of N_2, depends on the particular bacterial strain and on the carbon source. As ethylene concentrations as low as 10 ppb can elicit plant responses, any ethylene production by microbes could have profound consequences (Primrose and Dilworth 1991) especially because endophitic organism would release this plant regulator inside the plant.

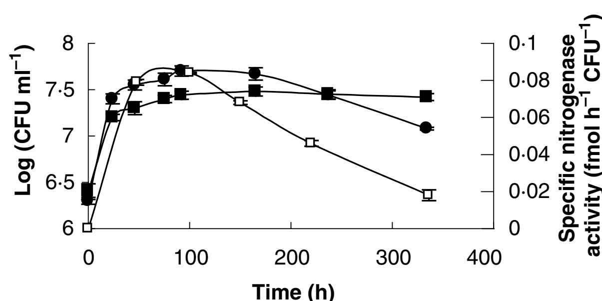

Fig. 1 Growth and specific nitrogenase activity curves in *Azospirillum* sp. grown in NFb_{Trp} (■ CFU; □ nitrogenase) and NFb_{TrpAS} (● CFU)

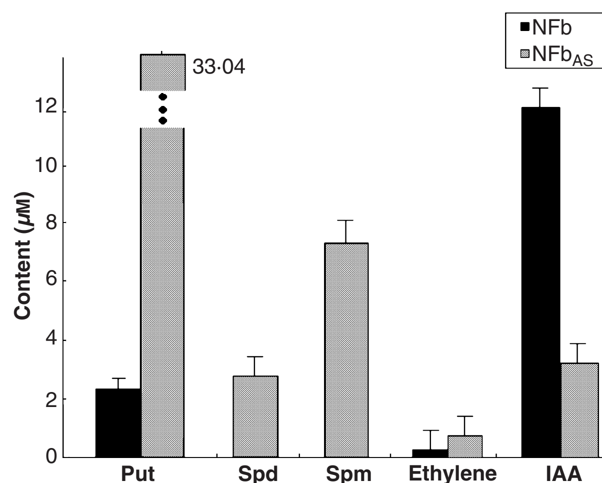

Fig. 2 Concentrations of free-polyamine, Indole-3-acetic acid (IAA) and ethylene released by a 250 h *Azospirillum* sp. culture grown in both NFb and in NFb_{AS}. Putrescine (Put), spermidine (Spd), spermine (Spm)

Although Barbieri *et al.* (1986) showed that IAA excretion is independent of nitrogen fixation, N_2-grown *Azospirillum* sp. cells showed to be better IAA producers than those grown in NH_4^+ (Fig. 2); however, these results are insufficient to propose that there might be a relationship between IAA synthesis and nitrogenase activity.

A higher level of IAA excretion was observed in NFb cultures, whereas higher levels of ethylene were excreted in NFb_{AS} cultures. These results do not reflect the intracellular situation, as described by Glick (1995), in which IAA stimulates ethylene synthesis.

Similarly to the results of Pati *et al.* (1994) and Hartmann *et al.* (1983) in which different types and quantities of amino acids were released by different diazotrophic bacteria, our experiments show that several amino acids were excreted by *Azospirillum*. Some of the amino acids we detected were also found by the authors referenced above. This paper also reports the release of amino acids in both media containing either N_2 or NH_4^+: five amino acids in either situation, only four in NFb_{AS}, and only one detected in NFb (Fig. 3). NH_4^+ availability in the culture medium had an impact on the

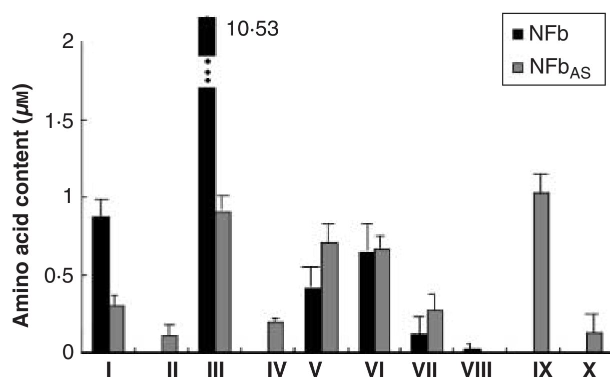

Fig. 3 Amino acids production (μM) by *Azospirillum*, in 240 h cultures, grown in both NFb and in NFb$_{AS}$. I, Glu; II, Asp; III, Ser; IV, Gln; V, Thr; VI, Ala; VII, Tyr; VIII, Leu; IX, Ile; X, Phe

diversity of the released amino acids but no reasonable explanation to such results has been found. Ruinen (1965) described the excretion of nitrogen-containing substances, especially amino acids, by bacteria, in the natural environment. However, to this day there is virtually no information about mechanisms through which this release occurs. Similarly, very little is known about the possible advantages of this release to the bacterium and about the type or quantity of amino acids excreted into culture media (Pati *et al.* 1994) or in nature. When in the endophytic state, *Azospirillum* might directly contribute to plant nutrition by liberating amino acids, whereas when in the free-living state, the released amino acids would enrich soils, becoming an indirect nutrient source to the plants.

Our results indicate that the excretion of the studied substances is independent of nitrogenase activity and suggest that the cells present differences in the regulation of several metabolic pathways as a function of the nitrogen source.

The analysed substances may be of great importance in the establishment of associations between plant and micro-organisms, other than suggest another potential property of members of the *Azospirillum* genus, the best characterized genus of plant-promoting bacteria. However, rhizobacteria, which can release plant growth promoting substances in laboratory, will not necessarily have any significant impact on plants in the natural environment.

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