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Gibberellin Production by *Azospirillum lipoferum* Cultured in Chemically-Defined Medium as Affected by Oxygen Availability and Water Status

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Abstract

Azospirillum growing in plant roots will experience varying water potentials and O_2 concentrations. Here we have investigated the effect of varying water potential or O_2 concentration on growth and gibberellin A_3 (GA_3) production of *Azospirillum* cultures. *Azospirillum lipoferum* strain USA 5b was grown in Nitrogen-free biotin medium (NFb) with malic acid (5 g l^{-1}) and NH_4Cl (1.25 g l^{-1}) as the C and N sources. Different water potentials (Ψ_w) in the media were made up by varying the NH_4Cl concentration, or with the addition of polyethylene glycol (PEG 8000). Different O_2 concentrations in the medium were obtained by varying the volumes of medium in 135 ml flasks with or without gas exchange. Gibberellin identity and quantity was assessed from the bacterial cultures by capillary gas chromatography-selected ion monitoring with [$^2H_2$]gibberellins as internal standards. Bacterial growth and viability was reduced by increasing the NH_4Cl concentration, increasing the PEG concentration, or restricting gas exchange. The total GA_3 (the main gibberellin identified from *Azospirillum* spp.) produced by each culture was reduced severely at high NH_4Cl or with restricted gas exchange. With PEG however, the total GA_3 was reduced by only 50% at the highest concentration (Ψ_w = –1.21 MPa) despite a 90% reduction in cell numbers, re-

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reflecting an increase in GA_3 produced per cell with increasing PEG concentration. This result helps to explain the positive effects of rhizospheric and endophytic *Azospirillum* spp. in alleviating the effects of water stress in infected plants through gibberellin production.

Keywords: Gibberellin, *Azospirillum lipoferum*, oxygen availability, water status

1. Introduction

Azospirillum spp. are soil rhizobacteria which grow either in the interface between the soil solution and the plant root or inside the plant itself (Patriquin et al., 1983), promoting growth and yield in cereals (Okon, 1985). Although their beneficial effects on growth were first attributed to N_2 fixation it has been established that the amount of N_2 fixed is too low to explain the increase in N uptake and assimilation by the plant (Boddey and Döbereiner, 1988). Thus, it has been proposed that the plant growth response might be caused by growth-promoting substances (Tien et al., 1979), especially gibberellins (GAs; Fulchieri et al., 1993; Lucangeli and Bottini, 1996) produced by bacteria. In fact, chemically-defined cultures of *A. lipoferum* produce indole-3-acetic acid (IAA, Crozier et al., 1988) and the GAs GA_1, GA_3, GA_5, GA_9 and GA_{20} (Bottini et al., 1989; Piccoli et al., 1996 and 1997), while *A. brasilense* cultures are capable of producing GA_1 and GA_3 (Janzen et al., 1992). This hormone production has been related to root growth promotion (Fulchieri et al., 1993) which in turn may improve water absorption and nutrient uptake (Kapulnik et al., 1985). Shoot growth is also enhanced by *Azospirillum* spp. in the dwarves *d1* of maize and *dx* of rice which are defective for the biosynthesis of active GAs, suggesting the *in vivo* production of these phytohormones by endophytic bacteria (Lucangeli and Bottini, 1996). Moreover, chemically-defined cultures of *A. lipoferum* are able to hydrolyze GA glucosyl conjugates both *in vitro* (Piccoli et al., 1997) and *in vivo* (Cassán et al., 1998), releasing GAs and so providing another way to promote plant growth (Fulchieri et al., 1993).

In chemically-defined culture media GA production by *A. lipoferum* is related to N availability, initial pH (Piccoli and Bottini. 1994) and light quality (Piccoli and Bottini. 1996). Thus it seems possible that in natural conditions GA production might also be affected by variation in these and other environmental factors, including O_2 concentration and water potential (Ψ_w).

A. brasilense in culture is capable of growing very well with high O_2 concentration if NH_4Cl is present, but in semi-solid medium without N the bacteria seek microaerobic conditions i.e. they develop as a film below the

surface or enter capillaries and move towards a decreasing gradient of O_2 concentration, and so protect nitrogenase activity (Okon et al., 1976). It is possible that bacteria inhabit microaerobic regions inside plant tissues, and so their viability and GA production should be checked under diverse O_2 conditions.

Azospirillum spp. in culture tolerate low Ψ_w imposed by polyethylene glycol (PEG), NaCl or sucrose, through osmoregulatory mechanisms (Hartmann, 1988). In plants *Azospirillum* confers resistance to the effects of water stress. For example, the slowing of the elongation rate of shoots of wheat grown in NaCl or PEG is significantly reversed by inoculation with *A. brasilense* (Creus et al., 1997). Such effects might be due partly to GAs produced by *Azospirillum*, since in *Zea mays A. lipoferum* inoculation or GA3 application significantly increases chlorophyll, K, Ca, soluble sugars and protein contents in plants grown at NaCl concentrations generating up to –1.2 MPa of osmotic strength, as compared with controls (Hamdia and Elkomy, 1998). Consequently, GA production of *Azospirillum* spp. should be checked under such adverse conditions.

Thus, the objective of the present work was to study the effects of O_2 availability and Ψ_w on GA production by *A. lipoferum* cultured in chemically-defined medium, in order to evaluate a possible role for GAs in alleviating the effects of water stress in plants infected with the bacterium.

2. Materials and Methods

Experiment 1. Media with different O_2 concentrations

To determine the effect of different O_2 concentrations on *Azospirillum lipoferum*, the strain USA 5b (kindly provided by Dr. V. Baldani, EMBRAPA, Itajaí, RJ, Brazil) was cultured in 135 ml flasks where the volume of NFb medium (nitrogen-free biotin [Bottini et al., 1989]; with malic acid 5 g l^{-1} and NH_4Cl 1.25 g l^{-1}) was varied. The volumes of medium used were 135, 100 or 50 ml, allowing air spaces of 0, 35 or 85 ml, respectively. Having in mind the possible effect of pH on culture growth and GA production, the 50 ml flasks were duplicated, one set containing 8 mM K_2HPO_4 and 8 mM KH_2PO_4 as buffers. In each case the volume of inoculum used was varied to give the same initial bacterial density in each culture, i.e., the volumes of *A. lipoferum* suspension (optical density at 540 nm, OD_{540} = 1.2) were 135, 100 and 50 µl, respectively. Two treatments were included; a) with gas exchange (the flasks had cotton lids); and b) without gas exchange (the flasks were sealed with Parafilm). The cultures were incubated until stationary phase at 32°C with continuous shaking (Lab-line orbit water bath, 80 rpm). In both treatments OD_{540} was measured

daily. pH and colony forming units (CFU) were measured every day in the gas exchange treatment, but in the Parafilm-sealed flasks were measured only at the beginning and at the end of the experiment to avoid O_2 exchange. At the end of the experiment, GA identity and content were assessed. The experiment was done in triplicate and data analyzed by ANOVA.

Experiment 2. Media with different NH_4Cl concentration

A. lipoferum strain USA 5b was grown in several 1000 ml flasks in 300 ml of NFb medium with malic acid (5 g l^{-1}) as the C source. The cultures were incubated until stationary phase at 32°C with continuous shaking (Lab-line orbit water bath, 80 rpm), and then OD_{540}, pH, CFU, and GA content were measured in experiments done in triplicate. Different water potentials (Ψ_w) in the solution media were generated by varying the NH_4Cl concentration (10, 7.5, 5.0, or 1.25 g l^{-1}). The NH_4Cl then served as both N source and osmoticum, avoiding the use of NaCl which would incorporate an additional factor, salinity, difficult to separate from the osmotic effect. Additionally, the objective was to change the Ψ_w of the solutions without changing either the chemical composition or the physical conditions of the media used in all the previous experiments on GA production by *A. lipoferum* (Bottini et al., 1989; Piccoli and Bottini 1994 and 1996; Piccoli et al., 1996 and 1997). The different Ψ_w values in the medium were assessed by direct measurement in a vapor pressure osmometer (Wescor 5500) previously calibrated with distilled water. The values from high to low concentration were -0.955, -0.852, -0.722, and -0.519 MPa. The data were analyzed by ANOVA.

Experiment 3. Media with different PEG concentration

A. lipoferum strain USA 5b was grown in 1000 ml flasks in 300 ml of NFb medium as experiment 2 (malic acid 5 g l^{-1} and NH_4Cl 1.25 g l^{-1} as C and N sources) with the addition of polyethylene glycol (PEG) 8000. Thus different Ψ_w in the solution media were generated by varying PEG (20, 15, 10, 5, or 0%). The different Ψ_w, assessed by measurement in the vapor pressure osmometer, were –1.21 –0.9, –0.79, –0.71 or –0.59 MPa, respectively. As in experiment 1, in order to check the possible influence of pH, 8 mM of K_2HPO_4 and 8 mM of KH_2PO_4 were added to two duplicate sets of the 0 and 10% PEG flasks. The resulting Ψ_w were –0.62 and –0.89 MPa, respectively. The cultures were incubated at 32°C with continuous shaking (Lab-line orbit water bath, 80 rpm) until stationary phase. The rest of the conditions and measurements were as in experiments 1 and 2.

GA evaluation

For gibberellin (GA) evaluation, the cultures (including liquid + bacteria) were sonicated, centrifuged at 10,000 g for 15 min, and the supernatant filtered under vacuo throughout 0.22 μm cellulose filters. After adding 100 ng of each of 17,17[$^2H_2$]-$GA_{(1/3/5/9/20)}$ (Prof. L. Mander, Australian National University) as internal standards, the filtrate was acidified to pH 3 and partitioned 4 times with 100 ml ethyl acetate saturated with aqueous 5% acetic acid. The ethyl acetate phase (containing free GAs) was evaporated under vacuum, dissolved in 10% aqueous methanol, filtered throughout 0.2 μm filters and submitted to reverse phase C_{18} HPLC (μ-Bondapak, 3.9 x 300 mm, Waters Associates column) in a Konik 500 (Konik Inc) system. Elution was done at 2 ml min^{-1} with a methanol gradient of 10 to 73% in 1% aqueous acetic acid. Thirty fractions of 4 ml were collected, dried and bioassayed in the dwarf rice cv. Tan-ginbozu micro-drop bioassay modified by Nishijima and Katsura (1989). Groups of fractions showing major bioactivity were then injected in HPLC with a NMe_2 (Nucleosil) column, eluted with 99.9% methanol in 0.1% acetic acid at 1 ml min^{-1} and bioassayed again. Bioactive fractions were grouped, then methylated with ethereal diazomethane and silylated with 1:1 pyridine:bis-trimethylsylil-trifluoroacetamide plus 1% trimethylchlorosylane (Pierce Chem. Co.). After dissolving in 10 μl of hexane, 1 μl was injected in a capillary gas chromatography-mass spectrometry (GC-MS) HP-5890 Series II GC-5970 MSD system (Hewlett-Packard Inc.). The GC column was a HP-5 (Hewlett-Packard 0.22 mm internal diameter, 30 m long, 0.25 μm film thickness) eluted with He (1 ml min^{-1}). The GC program was 100°C to 260°C at 20°C min^{-1}, then 10 min at 260°C. The interface temperature was 280°C, and data acquisition was controlled by a Vectra 300 Series computer. Six ions (3 for endogenous GAs, 3 for the deutero standards) plus ion 85 for an hydrocarbon mixture were scanned in SIM mode at a rate of 0.8 cycles sec^{-1}. A mixture of n-alkanes was co-injected in order to determine relative retention index according to Kovats (1958). Calculation of GA content was done by comparison of the peak area for the parent ion of [$^2H_2$]GAs with that of the endogenous compound, from samples of three experiments and data were analyzed by ANOVA.

3. Results and Discussion

The most abundant GA identified in all experiments was GA_3, confirming earlier results (Bottini et al., 1989; Janzen et al., 1992; Piccoli and Bottini, 1994 and 1996; Piccoli et al., 1996). Also notable was the finding of GA_1 at lower Ψ_w (–0.722 MPa, Table 4; 0.9 and 1.21 MPa, Table 6), although GA_3 was always more abundant. This confirms former results (Piccoli and Bottini, 1994 and 1996;

Table 1. OD_{540}, CFU ml^{-1} (10^{-7} dilution) and pH in cultures of *A. lipoferum* strain USA 5b, cultured with (W/E) and without (WT/E) gas exchange until stationary phase.

Volume (ml)	OD		CFU		Final pH	
	W/E	WT/E	W/E	WT/E	W/E	WT/E
135	0.08 b	0.02 c	10.0 c	5.4 c	7.2 b	7.35 b
100	0.70 a	0.22 b	114 b	60 b	7.9 b	7.65 b
50	0.96 a	0.90 a	220 a	104 a	9.1 a	8.70 a
50 *	0.81 a	0.39 b	140 b	60 b	7.2 b	7.0 b

Data are the means of three experiments. Values with different letters in the same column are significantly different (p 0.95). *8 mM K_2HPO_4 and 8 mM KH_2PO_4 were added to the culture medium.

Table 2. Quantification of GA_3 by GC-MS-SIM with $[^2H_2]GA_3$ as an internal standard expressed in relation to volume, OD_{540} and CFU (10^{-7} dilution) in cultures of *A. lipoferum* cultured with (W/E) and without (WT/E) gas exchange until stationary phase.

Volume (ml)	ng GA_3 ml^{-1}	ng GA_3 ml^{-1} OD^{-1}	ng GA_3 ml^{-1} CFU^{-1}
135 W/E	0.84 c	10.50	0.084
100 W/E	1.88 b	2.68	0.016
50 W/E	17.4 a	18.12	0.079
50 W/E*	5.8 b	7.16	0.041
135 WT/E	0.55 c	27.5	0.10
100 WT/E	0.84 c	3.82	0.014
50 WT/E	3.31 b	3.68	0.032
50 WT/E*	3.9 b	10.0	0.065

Data are the means of three experiments. Values with different letters in the same column are significantly different (p 0.95). *8 mM K_2HPO_4 and 8 mM KH_2PO_4 were added to the culture medium.

Table 3. OD_{540}, CFU (dilution 10^{-7}) and pH of *A. lipoferum* strain USA 5b cultures grown until stationary phase in NFb media with different Ψ_w generated by NH_4Cl.

Ψ_w	OD	CFU	Final pH
−0.955	0.87 b	29 c	8.3 a
−0.852	0.85 b	38 c	8.2 a
−0.722	0.93 b	122 b	8.3 a
−0.519	1.30 a	354 a	8.5 a

Data are the means of three experiments. Values with different letters in the same column are significantly different (p 0.95).

Table 4. Quantification of gibberellins (ng ml^{-1}) by GC-MS-SIM with [$^2H_2$]gibberellins as internal standards in cultures of *A. lipoferum* strain USA 5b grown until stationary phase in NFb media with different Ψ_w generated by NH_4Cl.

Ψ_w	GA_1	GA_3	ng GA_3 ml^{-1} OD^{-1}	ng GA_3 ml^{-1} CFU^{-1}
−0.955	nd	nd	nd	nd
−0.852	nd	tr	nd	nd
−0.722	2.2	29.8 a	32.04	0.24
−0.519	nd	27.0 a	20.76	0.07

Data are the means of three experiments. Values with different letters in the same column are significantly different (p 0.95). nd = not detected; tr = traces.

Table 5. OD_{540}, CFU (dilution 10^{-7}) and pH of *A. lipoferum* strain USA 5b cultures grown until stationary phase in NFb media with different Ψ_w generated by PEG.

Ψ_w	OD	CFU	Final pH
−1.21	0.15 d	12 e	9.95 a
−0.9	0.495 c	42 d	9.80 a
−0.89*	0.73 bc	160 bc	7.70 c
−0.79	0.90 b	100 c	9.45 b
−0.71	1.25 a	190 b	9.05 b
−0.63*	0.62 bc	140 c	7.15 d
−0.519	1.30 a	354 a	8.80 bc

Data are the means of three experiments. Values with different letters in the same column are significantly different (p 0.95). *8 mM K_2HPO_4 and 8 mM KH_2PO_4 were added to the culture medium.

Table 6. Quantification of gibberellins (ng ml^{-1} of culture) by GC-MS-SIM with [$^2H_2$]gibberellins as internal standards in cultures of *A. lipoferum* strain USA 5b grown until stationary phase in NFb media with PEG.

Ψ_w	GA_1	GA_3	ng GA_3 ml^{-1} OD^{-1}	ng GA_3 ml^{-1} CFU^{-1}
−1.21	1.0 a	14.5 c	96.66	1.20
−0.90	3.0 a	18.5 c	37.37	0.44
−0.89*	nd	6.0 d	8.2	0.03
−0.79	nd	68 a	75.5	0.68
−0.71	nd	38.5 b	30.8	0.20
−0.63*	nd	3.2 d	5.1	0.02
−0.519	nd	28.5 bc	21.9	0.08

Data are the means of three experiments. Values with different letters in the same column are significantly different (p 0.95). *8 mM K_2HPO_4 and 8 mM KH_2PO_4 were added to the culture medium. nd = not detected.

Piccoli et al., 1996) suggesting that in *A. lipoferum* GA_1 and GA_3 may come from different pathways. Also the GAs $GA_{5/9/20}$ were not found although they have been reported in former experiments (Piccoli et al., 1996 and 1997).

Restricting O_2 availability in the culture medium reduced bacterial growth and viability (Table 1) as expected (Okon et al., 1976), and also reduced total GA_3 production in all treatments (Table 2). With the exception of the most extreme of the treatments (that with no air space, and no gas exchange), reduced air space or restricted gas exchange tended also to reduce GA_3/OD or GA_3/CFU, which are estimates of average GA_3 production per unit number of cells or per viable cell (Table 2). The results suggest that GA_3 production is inhibited (on a per cell basis) by reduced O_2, consistent with the known function of GA biosynthetic enzymes as oxygenases (Kende and Zeevaart, 1997) and experiments with $^{18}O_2$ on GA_{20} metabolism in cotyledons of *Phaseolus vulgaris* (Smith et al., 1989). So, the results from the experiments with O_2 (Tables 1 and 2) showed that in order to obtain higher production of GAs, good aeration of the liquid culture system is needed with concomitant enhancement of microbial viability.

The results with the two lowest NH_4Cl concentrations showed that as the N concentration increased so did the GA_3/OD and GA_3/CFU ratios (Table 4). However at higher concentrations of N, while there was still rapid bacterial growth (Table 3), GA production was not detectable (Table 4). In fact N abundance inhibits GA formation in *A lipoferum* cultures (Piccoli and Bottini, 1994), as also seen in cultures of the fungi *Gibberella fujikuroi* (Candau et al., 1992) and *Fusarium moniliforme* (Rybakov and Bourd, 1991) where GA production begins once N is exhausted in the medium. From this point of view, the absence of GA production under high NH_4Cl might be in part a consequence of the higher N rather than any effect of further reduced Ψ_w.

The use of PEG instead of NH_4Cl as an osmoticum should obviate this problem. The addition of PEG up to a certain concentration increased total GA_3 produced, and this production then decreased with additional PEG (Table 6). However, GA_3/OD and GA_3/CFU in all cases increased over the control (no PEG). It has been claimed that PEG, often used to impose low water potentials (Ψ_w) in solution culture, decreases O_2 movement by increasing solution viscosity (Verslues et al., 1998) and so might have an additional effect through restricted O_2 availability. Comparison of these results with those of the oxygen experiment suggests that the effect of PEG on increasing GA_3/OD or GA_3/CFU is not due to O_2 depletion (which might be expected to reduce GA_3/OD or GA_3/CFU) and thus appears to be an effect of reduced Ψ_w. Up to a point, this will result in an increase in total GA produced, and this would seem to be the important issue as far as an *Azospirillum*-infected plant is concerned. While total GA production might decline thereafter with further reduced Ψ_w due to a reduction in the number and viability of cells (as reflected by OD_{540}

and CFU, Table 5), this might be partly offset by the increase in GA production per cell (as suggested by the GA_3/OD_{540} and GA_3/CFU results).

The initial pH of the cultures was always the same (6.8), and the final pH values reflected malic acid consumption and ammonia remaining in cultures growing at different rates. It is well known that in cultures of the fungi *Gibberella fujikuroi* (Candau et al., 1992) and *Fusarium moniliforme* (Rybakov and Bourd, 1991) GA production begins once N is exhausted in the medium, situation also found in *A. lipoferum* cultures (Piccoli and Bottini, 1994). Thus, N concentration and not final pH should be the main factor affecting GA production. In fact, from the experiment with variable concentrations of NH_4Cl (Table 3), it is possible to visualize that no significant changes in the final pH of the media were observed from the various treatments, although GA production was substantially different (Table 5). However, at higher PEG concentration pH could play an important role in order to explain that although the bacterial viability decreases strongly, this elevated pH might allow GA liberation out of the dead cells. The final pH in the media of PEG and O_2 experiments varied significantly (Tables 1 and 5), and this might affect GA production. For this reason additional treatments were included in the O_2 and PEG experiments where the pH influence on GA production was checked (Tables 1, 2, 5 and 6). By maintaining pH slightly over 7.0, there was a moderate reduction in culture growth (OD and CFU, Tables 1 and 5), and so was GA production under the less restrictive O_2 condition (50 W/E), while the amount of GA produced in restricted O_2 did not change (Table 2). However GA production was severely diminished when pH was kept around 7.0 in both cases, with or without PEG addition (Table 6). For some unknown reason, the presence of phosphates in the medium under no restrictive O_2 had a negative effect on the capacity of the bacterium for GA production.

From the above one might expect that reduced Ψ_w (up to a point) and high O_2 concentration inside the plant tissues infected by *Azospirillum* would favor GA production. By implication, *Azospirillum* spp. would play a significant role in water stress alleviation observed in infected plants (Creus et al., 1997; Hamdia and Elkomy, 1998) by enhancing GA production, perhaps through the stimulation of secondary metabolism as a sort of compensatory mechanism. O_2 is indispensable for *Azospirillum* growth, and low levels of oxygenation in the culture media could affect the biosynthetic pathways of secondary metabolites. GA biosynthesis requires O_2-dependent enzymes and so might also be modified. It is evident that in cultures which were more oxygenated and with reduced Ψ_w, GA production was increased (Tables 4 and 6).

In conclusion the results obtained regarding O_2 concentration and Ψ_w on GA production by *A. lipoferum* cultured in chemically-defined medium confirm the possible role of GAs in the promotion of plant growth by endophytic bacteria. The data obtained in this and previous work support the idea that the

beneficial effects of *Azospirillum* spp. in cereals might also be due to endophytic presence of the bacterium and not only to rhizospheric association (Lucangeli and Bottini, 1996). Moreover, the effect of *Azospirillum* spp. alleviating the effects of water deficits in cereal seedlings under salt and osmotic stresses (Creus et al., 1997; Hamdia and Elkomy, 1998) can be attributed at least partly to bacterial GA production. This would explain the better growth (greater fresh wt/dry wt) and faster elongation rate in shoots of inoculated plants. The effect might also be due to improvement of the active hairy root zone (Fulchieri et al., 1993), which will facilitate water absorption.

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REFERENCES

Boddey, R.M. and Döbereiner, J. 1988. Nitrogen fixation associated with grasses and cereals: recent results and perspectives for future research. *Plant and Soil* **108**: 53-65.

Bottini, R., Fulchieri, M., Pearce, D.W., and Pharis R.P. 1989. Identification of gibberellins A_1, A_3 and iso-A_3 in cultures of *Azospirillum lipoferum*. *Plant Physiology* **90**: 45–47.

Candau, R., Avalos, J., and Cerdá-Olmedo, E. 1992. Regulation of gibberellin biosynthesis in *Gibberella fujikuroi*. *Plant Physiology* **100**: 1184–1188.

Cassán, F., Masciarelli, O., and Bottini, R. 1998. Deconjugación de giberelinas *in vivo* por *Azospirillum* spp. en arroz enano. *Comunicaciones XXII Reunión Argentina de Fisiología Vegetal, Mar del Plata, Argentina*, pp. 308–309.

Creus, C., Sueldo, R., and Barassi, C. 1997. Shoot growth and water status in *Azospirillum*-inoculated wheat seedlings grown under osmotic and salt stresses. *Plant Physiological Biochemistry* **35**: 939–944.

Crozier, A., Arruda, P., Jasmin, J.M., Monteiro, A.M., and Sandberg, G. 1988. Analysis of indole-3-acetic acid and related indoles in culture medium from *Azospirillum lipoferum* and *Azospirillum brasilense*. *Applied and Environmental Microbiology* **54**: 2833–2837.

Fulchieri, M., Lucangeli, C., and Bottini, R. 1993. Inoculation with *Azospirillum lipoferum* affects growth and gibberellin status of corn seedling roots. *Plant and Cell Physiology* **34**: 1305–1309.

Hamdia, M.A. and Elkomy, H.M. 1998. Effect of salinity, gibberellic acid, and *Azospirillum* inoculation on growth and nitrogen uptake of *Zea mays*. *Biologia Plantarum* **40**: 109–120.

Hartmann, A. 1988. Osmoregulatory properties of *Azospirillum* spp. In: *Azospirillum IV, Genetics, Physiology, Ecology*. Klingmüller, W., ed. Springer-Verlag, Berlin, New York, pp. 122–130.

Janzen, R.A., Rood, S.B., Doormar, J.F., and Macgill, W.B. 1992. *Azospirillum brasilense* produces gibberellin in pure culture on chemically-defined medium and in co-culture with straw. *Soil Biological Biochemistry* **24**: 1061–1064.

Kapulnik, Y., Gafny, R., and Okon, Y. 1985. Effect of *Azospirillum* spp. inoculation on root development and NO_3^- uptake in wheat (*Triticum aestivum* cv. Miriam) in hydroponic systems. *Canadian Journal of Botany* **63**: 627–631.

Kende, H. and Zeevaart, J.A.D. 1997. The five "classical" plant hormones. *The Plant Cell* **9**: 1197–1210.

Kovats, E. 1958. Gas-chromatographische Charakterisierung organischer Verbindungen. Teil 1: Retention Indices aliphatischer Halogenide, Alkohole, Aldehyde und Ketone. *Helvetia Chimica Acta* **41**: 1915–1932.

Lucangeli, C. and Bottini, R. 1996. Reversion of dwarfism in *dwarf-1* maize (*Zea mays* L.) and *dwarf-x* rice (*Oryza sativa* L.) mutants by endophytic *Azospirillum* spp. *Biocell* **20**: 223–228.

Nishijima, T. and Katsura, M. 1989. A modified micro-drop bioassay using dwarf-rice for detection of femtomol quantities of gibberellins. *Plant and Cell Physiology* **30**: 623–627.

Okon, Y. 1985. *Azospirillum* as a potential inoculant for agriculture. *Trends in Biotechnology* **3**: 223-228.

Okon, Y., Albrecht, S.L., and Burris, R.H. 1976. Factors affecting growth and nitrogen fixation of *Spirillum lipoferum*. *Journal of Bacteriology* **128**: 592–597.

Patriquin, D.C., Döbereiner, J., and Jain, D.K. 1983. Sites and processes of association between diazotrophs and grasses. *Canadian Journal of Microbiology* **119**: 900–915.

Piccoli, P. and Bottini, R. 1994. Effect of C/N ratio, N content, pH, and incubation time on growth and gibberellin production by *Azospirillum lipoferum*. *Symbiosis* **17**: 229–236.

Piccoli, P. and Bottini, R. 1996. Gibberellin production in *Azospirillum lipoferum* cultures is enhanced by light. *Biocell* **20**: 185–196.

Piccoli, P., Lucangeli, C., Schneider, G., and Bottini, R. 1997. Hydrolysis of [17,17-$^2H_2$]gibberellin A_{20}-glucoside and [17,17-$^2H_2$]gibberellin A_{20}-glucosyl ester by *Azospirillum lipoferum* cultured in a nitrogen-free biotin-based chemically-defined medium. *Plant Growth Regulation* **23**: 179–182.

Piccoli, P., Masciarelli, O., and Bottini, R. 1996. Metabolism of 17,17-[$^2H_2$]-gibberellins A_4, A_9 and A_{20} by *Azospirillum lipoferum* in chemically-defined culture medium. *Symbiosis* **21**: 263–274.

Rybakov, Y. and Bourd, G. 1991. Nitrogen regulation of gibberellin biosynthesis enzyme complex in *Fusarium moniliforme*. *Journal of Biotechnology* **21**: 219–228.

Salisbury, F.B. and Ross, C.W. 1994. *Fisiología Vegetal, Segunda Edición Corregida*. Editorial Latinoamericana, México, pp. 827.

Smith, V.A., Albone, K.S., and MacMillan, J. 1989. Enzymatic 3β-hydroxylation of gibberellins A_{20} and A_5. In: *Gibberellins*. Takahashi, N. Phinney, B.O., and MacMillan, J., eds. Springer-Verlag, New York, Berlin, Heidelberg, pp. 62–71.

Tien, T.M., Diem, H.G., Gaskins, M.H., and Hubell, D.H. 1979. Plant growth substances produced by *Azospirillum brasilense* and their effect on the growth of pearl millet (*Pennisetum americanum* L). *Applied and Environmental Microbiology* **37**: 1016–1024.

Verslues, P., Ober, E., and Sharp, R. 1998. Root growth and oxygen relations at low water potentials. Impact of oxygen availability in polyethylene glycol solutions. *Plant Physiology* **116**: 1403–1412.