Pergamon

Soil Biol. Biochem. Vol. 29, No. 11/12, pp. 1743–1746, 1997

Printed in Great Britain
PII: S0038-0717(97)00066-7
0038-0717/97 $17.00 + 0.00

GROWTH AND NITRITE PRODUCTION BY *AZOSPIRILLUM* STRAINS SUBJECTED TO DIFFERENT LEVELS OF DISSOLVED OXYGEN IN THE MEDIUM

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(*Accepted 21 February 1997*)

Summary—*Azospirillum* growth responses to temperature and the capacity of NO_3^--grown bacteria to produce nitrite, were investigated in three southern Brazilian isolated strains exposed to various concentrations of dissolved oxygen in the medium. The strains *Azospirillum brasilense* Sp245 and JA04, and *A. lipoferum* JA03, responded differently to incubation temperature, strain JA03 showing more active growth at the lowest temperature, 28°C, while strains Sp245 and JA04 greater cell multiplication at 32 and 37°C. The exposure of bacteria to different amounts of oxygen dissolved in the medium permitted discrimination of the three strains with respect to growth and nitrite production. Strain JA03 showed remarkably high nitrite accumulation and more intense growth at the lowest oxygen tensions compared with strains Sp245 and JA04. It is conceivable that the highest growth measured in strain JA03 under semi-anaerobic conditions was partly due to efficient utilization of nitrate in respiration. Our data for *Azospirillum* suggest that the capacity of strain JA03 to withstand reduced oxygen concentrations and relatively lower temperature conditions during growth might be identified as favorable adaptive attributes leading to better acclimation to water-logged and highly compacted sub-tropical soils. © 1997 Published by Elsevier Science Ltd

INTRODUCTION

The genus *Azospirillum* is probably one of most extensively studied plant-growth promoting rhizobacteria non-symbiotically associated with roots, particularly in cereals and grasses (Bashan and Levanony, 1990). These bacteria occur abundantly in tropical soils and have been identified on root surfaces and in intercellular spaces, primarily in the radicle elongation zone (Okon and Kapulnik, 1986; Bashan *et al.*, 1990). Experimental evidence obtained in Rio Grande do Sul, Brazil, has revealed that the association of the soil-borne *Azospirillum* with wheat roots enhanced plant growth (Didonet, unpub. data).

Numerous studies on the effects of plant–*Azospirillum* associations affecting root morphology have pointed out the involvement of hormone-like compounds produced by bacteria (Zimmer *et al.*, 1988; Bothe *et al.*, 1992; Dubrovsky *et al.*, 1994). The results obtained with wheat indicated that stimulation of root growth by exogenous nitrite or endogenous amounts produced by bacterial nitrate respiration (Bothe *et al.*, 1981), was supposedly due to the nitrite phytohormonal effect (Didonet and Magalhães, 1993). Didonet and Magalhães (1993) suggested that the capacity of the bacteria to excrete nitrite into the external medium might be enhanced under conditions of limiting oxygen. Indeed, in water-logged and compacted soils, nitrite was shown to accumulate in the rhizosphere in concentrations varying from 3 mM to 6 mM, depending on the strain and the assay conditions (Tibelius and Knowles, 1984; Stephan *et al.*, 1984). Data presented by Neuer *et al.* (1985) showed that in suspension cultures *Azospirillum* excreted appreciable amounts of nitrite produced by nitrate respiration. Experimental results obtained with cereals demonstrated that *Azospirillum* strains are predominantly nitrite reductase negative (nir^-) (Patriquin *et al.*, 1983). Bashan and Levanony (1989) reported that the adsorption of bacteria to wheat roots under microaerophilic conditions depended on the number and metabolic activity of bacteria present in the inoculum. It was established that metabolically-active bacteria are required for nitrite production under O_2 limiting conditions for the roots.

Despite the extensive research on the beneficial effects of root-growth promoting *Azospirillum*, we believe that some questions are still open for exper-

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imentation due to the variable results reported for different *Azospirillum* species and strains concerning their responses to the physical environment.

We have evaluated the growth responses of three *Azospirillum* strains: Sp245, JA04 and JA03, to different concentrations of dissolved oxygen in the medium, and to assess the extent to which these conditions affect the capacity of the bacteria to produce nitrite in a nitrate assay medium.

MATERIALS AND METHODS

The strains of *Azospirillum* utilized in this study were: (a) *A. brasilense* strain Sp245, isolated from chloramine-T surface-sterilized wheat roots, resistant to 20 μg streptomycin ml^{-1}; (b) *A. brasilense* strain JA04, isolated from washed wheat roots taken from a low nitrogen soil in Passo Fundo RS, Brazil; and (c) *A. lipoferum* strain JA03 isolated as in (b).

Experimental procedure and assays

The inoculum was obtained from isolated colonies of bacteria grown in Petri dishes containing potato–agar medium (Döbereiner, 1980). Two to three colonies were suspended and placed to grow in liquid NFb medium containing KNO_3 (2 g l^{-1}) as the nitrogen source. The cultures were harvested by centrifugation, washed three times in sterile water and diluted to the required cell concentration. The cell number was determined by correlating optical density at 660 nm, and the number of colonies formed ml^{-1} potato–agar medium.

Different initial amounts of dissolved oxygen were imposed by agitation of the medium for 1 h, in a rotary shaker adjusted to 60, 100, 150 or 200 rev min^{-1}, in addition to 60 rev min^{-1} plus N_2 purging for 5 min at 30°C. Measurements of oxygen concentration were carried out with a polarographic electrode immediately before bacterial inoculation. Nitrite content in the medium was determined colorimetrically by the method of Nicholas and Nason (1957) after removal of bacteria by centrifugation with a table-top centrifuge. The data are expressed as μg of nitrite ml^{-1} culture medium.

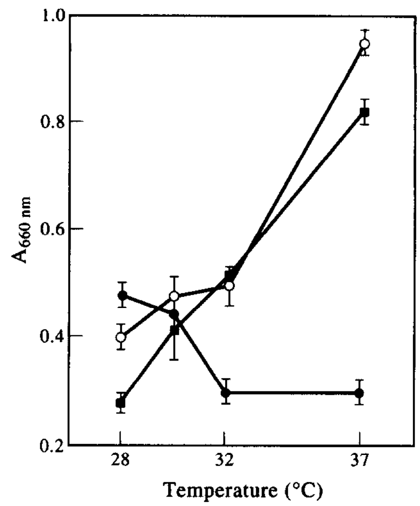

Fig. 1. The effect of temperature on growth of *Azospirillum* strains: Sp245 (■); JA04 (○); JA03 (●). Bacteria were incubated for 8 h in NFb medium plus 1 g NH_4Cl l^{-1}, at 200 rev min^{-1} agitation. Vertical bars indicate SD of the mean (n = 4).

RESULTS AND DISCUSSION

Measurements of the temperature effects on growth of *Azospirillum* strains showed comparatively higher rates of multiplication in strain JA03 at 28°C, the lowest incubation temperature (Fig. 1). At 30°C the three strains expressed similar growth responses, while at 32°C and 37°C strains JA04 and Sp245 presented more active growth after incubation for 8 h as compared with JA03. In contrast to *Azospirillum* species isolated from roots of tropical species, known for their adaptation to high temperatures, *A. lipoferum* strain JA03 seemed better acclimated to lower temperatures between 28°C and 30°C.

The cumulative growth of *Azospirillum* strains subjected for 24 h to different initial amounts of dissolved oxygen in the medium is shown in Table 1.

Table 1. Effect of different levels of dissolved oxygen on growth and nitrite accumulation in *Azospirillum* strains grown for 24 h at 30°C

Dissolved oxygen (cm^3 m^{-3})	Incubation speed (rev min^{-1})	*Azospirillum* strain[a]					
		Sp 245		JA 04		JA 03	
		Growth (A660 nm)	Nitrite (μg ml^{-1})	Growth (A660 nm)	Nitrite (μg ml^{-1})	Growth (A660 nm)	Nitrite (μg ml^{-1})
5.48 (±0.09)	60 + N$_2^b$	0.102 (±0.031)	ND	0.163 (±0.038)	0.73 (±0.03)	0.281 (±0.067)	515.75 (±3.01)
5.53 (±0.08)	60	0.165 (±0.041)	0.20 (±0.02)	0.140 (±0.063)	ND	0.623 (±0.053)	794.12 (±25.27)
6.87 (±0.10)	100	0.348 (±0.023)	1.67 (±0.05)	0.401 (±0.070)	2.53 (±0.61)	0.466 (±0.065)	740.66 (±108.30)
7.44 (±0,07)	150	0.594 (±0.015)	1.08 (±0.07)	0.566 (±0.018)	18.37 (±2.04)	0.605 (±0.077)	37.52 (±5.16)
7.66 (±0.08)	200	0.594 (±0.063)	6.35 (±0.71)	0.475 (±0.054)	16.20 (±0.26)	0.431 (±0.081)	0.44 (±0.08)

[a]The bacteria were grown in 100 ml of NFb medium, plus 2 g KNO_3 l^{-1} for 24 h at 30°C. Mean (n = 4) ± SD for each treatment.
[b]60 rev min^{-1} plus N_2 purging for 5 min.
ND, Not detected.

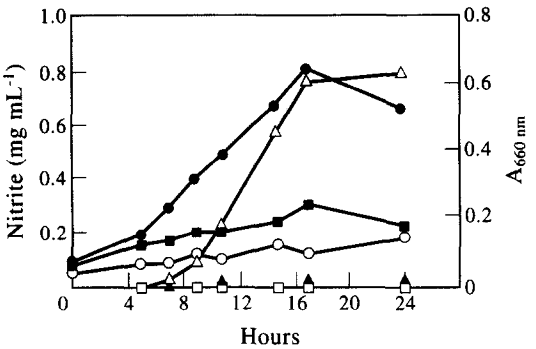

Fig. 2. Time course of growth and nitrite accumulation in *Azospirillum* strains incubated in NFb medium plus 1 g $KNO_3\ l^{-1}$. Strain Sp245: growth (■), nitrite (□); strain JA04: growth (○), nitrite (▲); strain JA03: growth (●), nitrite (△).

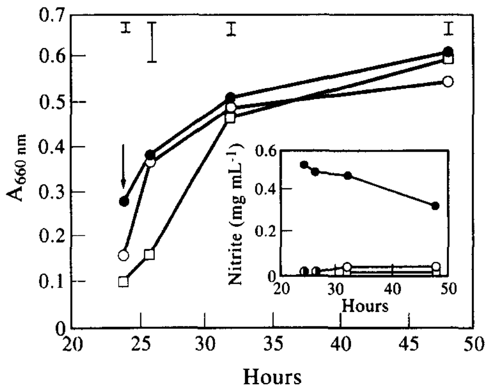

Fig. 3. Time course of growth and nitrite accumulation (insert) in *Azospirillum* strains incubated in NFb medium plus 2 g $KNO_3\ l^{-1}$, at 30°C. Bacteria were previosly exposed to agitation at 60 rev min^{-1} plus N_2 purging for 24 h and then transferred to 200 rev min^{-1} agitation for additional 24 h (arrow). Strain Sp245 (□); strain JA04 (○); strain JA03 (●). Vertical bars indicate SD of the mean (n = 4).

Our results demonstrate that incubation of bacteria in NFb medium plus 2 g $KNO_3\ l^{-1}$ caused promotive growth response in strain JA03 relative to strains JA04 and Sp245 on exposure to lower concentrations of oxygen. As the amount of dissolved oxygen in the medium increased the differences in growth among the strains practically disappeared, the pattern even being reversed at the highest concentration of oxygen present in the medium. Apparently, the highest cumulative growth observed in strain JA03 at lower oxygen contents was associated with the greater capacity of JA03 to utilize nitrate in respiration and to support higher concentrations of nitrite accumulated in the medium. Indeed, literature reports have shown that both *A. brasilense* and *A. lipoferum* species include denitrifying (nir$^+$) and non-denitrifying (nir$^-$) strains, that are capable of reducing nitrate to nitrite in the nitrate reductase reaction (Neyra and Dobereiner, 1979). In denitrifying strains metabolism of nitrite involves a dissimilatory nitrite reductase that catalyzes the reduction of nitrite to N_2O, leading to the production of gaseous NO and finally to N_2 (Dannberg *et al.*, 1989; Voswinkel *et al.*, 1991). In fact, among the strains used in this work JA03 was identified as the most active denitrifying strain (Neyra *et al.*, 1977). The data reported in Table 1 support the contention that strain JA03 was able to produce remarkably high amounts of nitrite, ca. 13 mM in 24 h, when exposed to semi-anaerobic conditions. Conversely, strains JA04 and Sp245 accumulated only traces of nitrite under the same oxygen concentrations.

The time-course data presented in Fig. 2 showed more active cell growth and higher nitrite accumulation in strain JA03 compared with JA04 and Sp245 over the 24 h period of incubation in NFb $+2\ g\ l^{-1}$ KNO_3 medium at a low oxygen concentration (60 rev min^{-1} agitation). Maximum concentration of nitrite was measured after incubation for 17 h, which coincided with the exponential phase of bacterial growth. The observed decrease in absorbance of JA03 medium in the period extending from 17 to 24 h of incubation was conceivably due to flocculation, a condition that has been reported in bacteria subjected to stress (Lamm and Neyra, 1981; Sadasivan and Neyra, 1987). In our assays, stress might have been due to low oxygen availability associated with a high bacterial population. When agitation of the medium was increased to 200 rev min^{-1}, the cell number progressively increased in the three strains from 24 to 32 h of incubation, reaching approximately the same population at the end of the experiment (Fig. 3). During the course of the experiment, nitrite content in the medium decreased from 520 to 325 $\mu g\ ml^{-1}$ in strain JA03, while only very small changes were observed in strains JA04 and Sp245 (Fig. 3, insert). This fall in nitrite accumulation might have been caused by an increased oxygen concentration associated with lower denitrification, by inhibition of dissimilatory nitrate reductase activity or, alternatively due to nitrite consumption as a source of nitrogen for bacterial growth.

Our data suggest that the greater capacity of *A. lipoferum* strain JA03 to grow in a semi-anaerobic environment, and the reported ability of these bacteria to utilize nitrate in respiration might confer on them favorable adaptive characteristics to withstand water-logged or highly compacted soils, conditions that have been frequently reported in heavily cropped cereal fields in southern Brazil.

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