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Response to oxygen of diazotrophic *Azospirillum brasilense* — *Arthrobacter giacomelloi* mixed batch culture

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Abstract. *Azospirillum brasilense* and *Arthrobacter giacomelloi* were grown together in batch culture under different oxygen pressures. The response to oxygen of growth, nitrogenase activity and respiration rate was determined. The two microorganisms were found to be able to coexist all over the range of partial oxygen pressures examined, that is from 0.004–0.20 bar. Nitrogenase activity by mixed culture of *A. brasilense* and *A. giacomelloi* always appeared higher than that of *A. brasilense* pure culture. Low respiratory activity at partial oxygen pressures higher than 0.02 bar by both pure and mixed cultures seemed not to account for the high nitrogenase activity and improved oxygen tolerance of the mixed culture.

Key words: *Arthrobacter giacomelloi* — *Azospirillum brasilense* — Mixed culture — Nitrogenase activity — Oxygen tolerance

The physiology, the ecology and the applications in agriculture of diazotrophic *Azospirillum* species are well known (Okon 1982, 1985). However, the positive results obtained by using *Azospirillum* as inoculant for cereal species led to the problems of survival and competitive ability of this microorganism in the rhizosphere system. In fact bacteria rarely occur in nature as monocultures and the behaviour of a species as a member of a community can be different from its behaviour as an axenic population.

Nitrogen fixation is the result of combined activities of different groups of rhizosphere microorganisms and it is important to give great attention to parameters that regulate interactions within a microbial community.

Positive interactions, resulting in increased nitrogenase activity, were reported by Jagnow (1983) in model experiments with pure and mixed cultures of *Azospirillum lipoferum, Klebsiella* sp. and *Enterobacter* sp. and by Halsall and Goodchild (1986) who demonstrated a physical association between *Cellulomonas* sp. and *Azospirillum brasilense* when grown on straw and on pure cellulose. Previously we described a stable coexistence and an increased nitrogenase activity in a mixed culture of *Azospirillum brasilense* and *Arthrobacter giacomelloi* grown under different cultural conditions (Cacciari et al. 1984, 1986).

As nitrogen fixation activity of aerobes is strictly dependent on oxygen availability (Drodz and Postgate 1970; Cacciari et al. 1979; Okon et al. 1976, 1977) the present study was undertaken to evaluate the response to oxygen of a mixed culture of *A. brasilense* and *A. giacomelloi* compared with that of pure cultures of the same microorganisms.

Materials and methods

Organisms and culture conditions. Azospirillum brasilense Cd (ATCC 29710) and *Arthrobacter giacomelloi* (DSM 3681) were grown at 30°C in a medium (SuB_7^-) containing 1.77 g K_2HPO_4, 0.68 g KH_2PO_4, 0.14 g NaCl, 0.132 g $CaCl_2$, 0.2 g $MgSO_4 \cdot 7\ H_2O$, 5.0 g sodium succinate, 0.08 g yeast extract, 40 µg biotin, 2.5 mg $FeSO_4 \cdot 7\ H_2O$, 2.9 mg H_3BO_3, 1.2 mg $CoSO_4 \cdot 7\ H_2O$, 0.1 mg $CuSO_4 \cdot 5\ H_2O$, 0.09 mg $MnCl_2$ $4\ H_2O$, 2.5 mg $NaMoO_4 \cdot 2\ H_2O$, 1.2 mg $ZnSO_4 \cdot 7\ H_2O$ in 1 l of distilled water, pH = 7.0. Viable cell numbers were determined by plate counts using nutrient agar medium.

Single and mixed batch cultures (100 ml of SuB_7^- medium) were grown for 48 h in a system of three vessels with the same magnetic stirring (100 rev/min) and aeration (100 ml/min). Different partial oxygen pressures (pO_2) were tested: 0.004, 0.006, 0.01, 0.02, 0.05, 0.10, 0.20 bar. The desired gas mixture was achieved by passing nitrogen and air at the same pressure from high pressure cylinders through a gas mixing pump (H. Wösthoff, Bochum, FRG, Typ 1M300/a) that was connected with the vessels.

Pure cultures were inoculated with 5 ml of overnight cultures obtained from a colony picked up from nutrient agar plate. The mixed culture was inoculated with 5 ml of each pure culture.

Growth curves. The growth of both pure and mixed cultures was followed and continuously recorded using a turbidometric apparatus. The doubling time (t_d) of cultures was

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Abbreviations: pO_2, partial oxygen pressure

measured during the logarithmic phase of growth and the maximum specific growth rates (μ_{max}) were calculated from the equation:

$$\mu = \frac{\ln 2}{t_d}.$$

Nitrogenase and respiratory activities. Nitrogenase and respiratory activities were determined after 24 and 48 h of growth. 5-ml culture samples were collected by a sterile syringe and directly injected into vials in the presence of argon and acetylene (10%) at the same oxygen pressure as during growth.

Duplicate samples were incubated at 30° C and the ethylene produced was determined with a gas chromatograph (Perkin-Elmer 900, Porapak N-column, f.i.d.).

The oxygen uptake of cultures was measured at 30° C in samples rapidly transferred to the respiration vessel of a Beckman Model 0260 Oxygen Analyzer as previously described (Cacciari et al. 1986).

Analyses. The protein content of cultures was determined by the method of Lowry et al. (1951), using bovine serum albumin as standard. Bacterial dry weight was determined in duplicate by centrifuging and washing 10-ml culture samples and drying the cells to constant weight at 95° C. Succinate consumption was estimated by the enzymatic method of Williamson (1963). The total biomass growth yield was expressed as milligrams dry weight per gram of succinate consumed.

Statistics. Data collected from two experiments, each with duplicate samples, are presented as the means ± the standard error.

Results and discussion

Azospirillum brasilense and *Arthrobacter giacomelloi* were able to grow and establish a positive interaction in a co-culture under different partial oxygen pressures.

However, (Fig. 1) the number of cells of both bacteria were generally lower in the mixed culture, even though the inoculum size was the same as that of the pure cultures. *A. giacomelloi* culture showed higher viable cell numbers at pO_2 of 0.02 and 0.05 bar while the population size of *A. brasilense* pure culture reached a maximum at pO_2 of 0.006 bar, thus confirming previous results obtained in batch culture (Cacciari et al. 1986). A similar behaviour of *A. brasilense* was not found in the mixed culture where the number of viable cells decreased throughout with increasing partial oxygen pressures.

The ratio between *A. brasilense* and *A. giacomelloi* ranged from 1 − 0.16. It was inversely related to oxygen pressure and seemed to reflect a major sensitivity of *A. brasilense* to increasing oxygen concentrations.

Culture growth rates, dry weights and growth yields (Table 1), behaved similarly to viable cell numbers. *A. brasilense* under nitrogen-fixing conditions was strictly dependent on pO_2 and exhibited the highest μ_{max} at pO_2 of 0.006 bar in agreement with the results reported by Okon et al. (1977). *A. giacomelloi* growth rate reached maximum value at pO_2 of 0.02 bar while the mixed culture showed higher μ_{max} at pO_2 0.01 bar. Though in pure as well as in

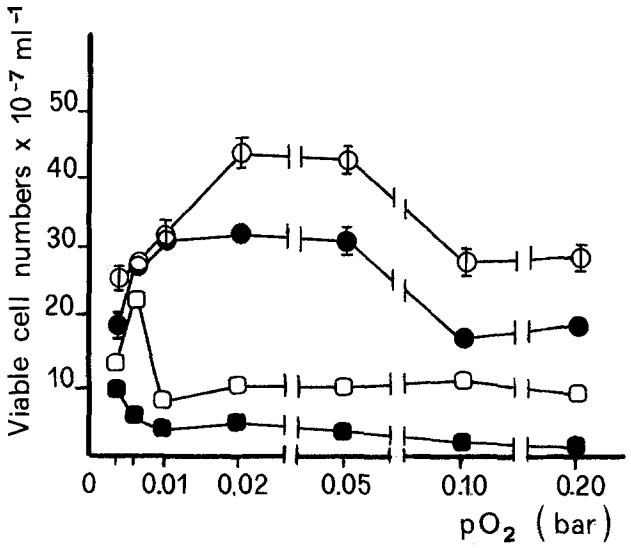

Fig. 1. Viable cell numbers of *Azospirillum brasilense* Cd (□, ■) and *Arthrobacter giacomelloi* (○, ●) pure (□, ○) and mixed (■, ●) batch cultures grown for 48 h at different partial oxygen pressures. Each bar represents the mean ± standard error; in some cases the SE in within the dimensions of the symbols

mixed cultures the maximum values of growth rate were obtained in the same narrow range of pO_2, the mixed culture appeared to be more oxygen tolerant at oxygen pressures greater than 0.02 bar. Values of the biomass yield revealed that at the lowest pO_2 examined both *A. brasilense* and mixed cultures showed a greater growth efficiency than *A. giacomelloi* culture.

Protein content of *A. brasilense* culture did not exceed 25% of dry weight at optimal pO_2, while values of about 18% of dry weight were maintained by both *A. giacomelloi* and mixed cultures all over the range of pO_2 examined. The biomass yield and the protein content of succinate-grown *A. brasilense* were lower than those reported for malate-grown cells (Hurek et al. 1988). These results may be due to the nature of substrate and the amount of phosphate in the medium used (Day and Döbereiner 1976; Okon et al. 1976).

Nitrogenase and respiratory activities of samples from *A. brasilense* and *A. giacomelloi* pure and mixed cultures were examined after 24 and 48 h of growth (Fig. 2a, b), regardless the growth phase of the cultures. As it is generally reported (Okon et al. 1976, 1977; Nelson and Knowles 1978; Kloss et al. 1983; Hartmann et al. 1985), nitrogenase activity of *A. brasilense*, was optimal at pO_2 lower than 0.01 bar, then declining sharply just at pO_2 of 0.02 bar. Our results confirmed these findings although low but detectable values of nitrogenase activity were obtained also in the range of pO_2 between 0.02 and 0.2 bar. Nitrogenase activity of *Arthrobacter* species is generally low (Cacciari et al. 1979, 1986). The values of ethylene produced by *A. giacomelloi* (not depicted in graph) ranged from 40 − 360 nmol h^{-1} mg^{-1} protein with the maximum at pO_2 of 0.1 bar in the cells grown for 24 h. The 48 h-old culture samples reached maximum nitrogenase activities (215 nmol ethylene formed h^{-1} mg^{-1} protein) at pO_2 of 0.02 bar. Acetylene reduction by samples from mixed culture exhibited curves similar in shape to those of *A. brasilense* either after 24 or 48 h of

Table 1. Maximum specific growth rates (μ_{max} in h^{-1}), dry weights (D.W. in $\mu g\ ml^{-1}$) and growth yields (Y in mg D.W. g^{-1} of succinate consumed) of *Azospirillum brasilense* Cd and *Arthrobacter giacomelloi* pure and mixed batch cultures grown for 48 h at different partial oxygen pressures (pO_2 in bar)

pO_2	*Azospirillum brasilense* Cd			*Arthrobacter giacomelloi*			Mixed culture		
	μ^a_{max}	D.W.[b]	Y[b]	μ^a_{max}	D.W.[b]	Y[b]	μ^a_{max}	D.W.[b]	Y[b]
0.004	0.064	193 ± 9	103 ± 5.1	0.053	133 ± 6	71 ± 3.5	n.d.[c]	160 ± 9	89 ± 4.9
0.006	0.100	220 ± 16	118 ± 6.6	0.070	130 ± 9	73 ± 4.6	0.056	166 ± 10	98 ± 4.4
0.01	0.070	120 ± 7	65 ± 3.7	0.085	130 ± 10	72 ± 4.4	0.092	130 ± 8	71 ± 3.7
0.02	0.059	117 ± 8	62 ± 3.0	0.098	146 ± 11	78 ± 3.8	0.086	142 ± 8	76 ± 4.1
0.05	0.047	140 ± 10	76 ± 4.7	0.067	150 ± 9	82 ± 5.4	0.072	153 ± 9	81 ± 4.3
0.1	0.030	145 ± 9	77 ± 4.4	0.044	120 ± 9	65 ± 4.2	0.064	107 ± 8	57 ± 3.0
0.2	0.018	140 ± 9	75 ± 4.7	0.034	123 ± 9	65 ± 3.9	0.064	115 ± 8	62 ± 3.5

[a] Data were significantly different, at a level of 99% in the range of pO_2 between 0.01 and 0.2 bar (statistical analysis based on a two-way analysis of variance)
[b] Data are given as means ± standard error
[c] Not determined

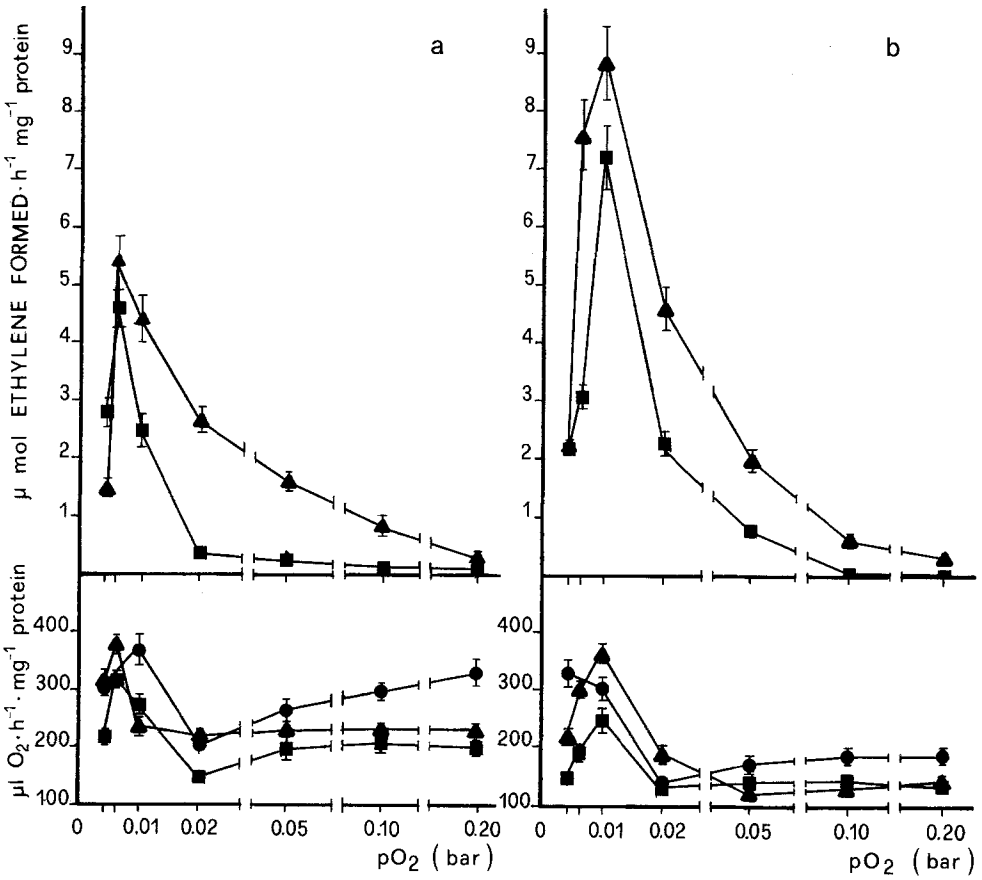

Fig. 2a, b
Nitrogenase activity and oxygen uptake by *Azospirillum brasilense* Cd and *Arthrobacter giacomelloi* pure and mixed cultures grown at different partial oxygen pressures. Acetylene reduction activity was detected in samples incubated at the same pO_2 as during growth. *Symbols:* ■ *A. brasilense*; ● *A. giacomelloi*; ▲ mixed culture. **a** Samples after 24 h of growth. **b** Samples after 48 h of growth. Each bar represents the mean ± standard error; in some cases the SE is within the dimensions of the symbols

growth (Fig. 2a, b). However, despite the decrease in *A. brasilense* cell number and the very low nitrogenase activity by *A. giacomelloi*, the mixed culture samples exhibited a greater nitrogenase activity over the whole range of pO_2 examined, reaching at pO_2 between 0.02 and 0.2 bar values much higher than that of *A. brasilense*.

In samples from *A. brasilense* culture grown for 24 h (Fig. 2a), the oxygen uptake rate showed a maximum at pO_2 of 0.006 bar and the values of oxygen consumption expressed on protein basis were within the range reported (Nelson and Knowles 1978; Kloss et al. 1983). After 48 h of growth (Fig. 2b) respiration rate showed a maximum at pO_2 of 0.01 bar. Oxygen uptake rate of *A. giacomelloi* (Fig. 2a, b) was high at the lowest pO_2 examined, decreased at pO_2 of 0.02 bar and increased again thereafter, mainly in the samples grown for 24 h.

High values of oxygen uptake at very low oxygen pressures have been reported not only in nitrogen-fixing con-

ditions but also in the presence of ammonium, either in *Azospirillum* (Nelson and Knowles 1978; Nur et al. 1982; Del Gallo et al. 1988) or in *Arthrobacter* (Cacciari et al. 1979, 1985), thus appearing not to be strictly related to diazotrophic conditions.

This response to oxygen of respiration rate of *A. brasilense* and *A. giacomelloi* appears different from that of *Azotobacter chroococcum* which is described to compensate for O_2 sensitivity through an increased respiratory activity (Drozd and Postgate 1970). Nelson and Knowles (1978) did not observe any evidence of a respiratory protection in *A. brasilense* while the occurrence of a respiratory type of protection has been proposed by Kloss et al. (1983) in *A. brasilense* and not excluded by Hurek et al. (1987) in *Azospirillum* strains isolated from the Kallar grass. Respiratory protection was shown not to be operative also in *Arthrobacter* (Cacciari et al. 1979).

The respiratory activity of samples from the mixed culture was in response to oxygen very similar to that of *A. brasilense*. Comparable low values of oxygen uptake were obtained in the range of pO_2 between 0.002 and 0.02 bar; nevertheless, the nitrogenase activity was much greater. As a consequence, these results do not seem to support the hypothesis of a respiratory protection in this mixed culture, under the present experimental conditions, as suggested in other co-cultures (Harper and Lynch 1984; Love and Rawson 1986; Veal and Lynch 1987).

However, the nature of interactions between *A. brasilense* and *A. giacomelloi* deserve further investigation to define the mechanisms by which the mixed culture can increase its nitrogenase activity.

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