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Growth and survival of *Azospirillum brasilense* and *Arthrobacter giacomelloi* in binary continuous culture*

I. CACCIARI, M. DEL GALLO**, S. IPPOLITI, D. LIPPI, T. PIETROSANTI and W. PIETROSANTI
Plant Radiobiochemistry and Ecophysiology Institute, National Research Council, Research Area, 00016 Monterotondo Scalo, Rome, Italy

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Summary *Azospirillum brasilense* and *Arthrobacter giacomelloi* were grown in single and mixed succinate-limited continuous cultures at a partial oxygen pressure of 0.01 atm. Growth, viability and survival during nutrient starvation were examined at various dilution rates. At $D = 0.05\ h^{-1}$, K_s values for succinate consumed were calculated. *Arthrobacter giacomelloi* viability was inversely related to dilution rate whereas *Azo. brasilense* was directly related. Slightly lower values of viability were obtained in mixed culture, but the ratio between the microorganisms was constant. The survival of *Arth. giacomelloi* in single culture decreased with increasing growth rate while survival of *Azo. brasilense* was directly related to dilution rate. Acetylene reduction activity was generally very low in both single and mixed cultures. Respiration rate was also determined and the mixed culture showed an oxygen uptake rate higher than that of single cultures.

Introduction

In recent years many studies have been concerned with the physiology, ecology and potential use in agriculture of bacteria of the genus *Azospirillum*[10,17,19,21]. When inoculated into a rhizosphere the population of azospirilla can be influenced by: (a) physical and chemical conditions of soil; (b) amounts and nature of organic material liberated by roots; (c) interactions with other microorganisms, and (d) predatory microfauna.

The purpose of the present work was to study some aspects of interaction between a strain of *Azospirillum brasilense* and a nitrogen-fixing strain of *Arthrobacter*. Bacteria of the genus *Arthobacter* are very numerous in soil and are typical autochthonous microbes. The interaction between these microorganisms has been studied in the chemostat, a technique that has proved to be of particular value in identifying parameters influencing bacterial components of mixed culture systems and in recognizing interactions within a microbial community[9,14,20].

This paper concerns the effect of dilution rate on growth, viability and survival during starvation of *Arth. giacomelloi* and *Azo. brasilense*

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** Dipartimento di Biologia Vegetale, Università di Roma, Largo Cristina di Svezia, 24–00165 Rome, Italy.

grown in single and mixed cultures. To ascertain whether competition between these bacteria could occur, the cultures were grown under succinate limitation in binary continuous culture according to the method described by Kemp *et al.*[11]. We have used the binary continuous culture method to create a simplified model of the rhizosphere environment and to study, in both single and mixed culture, the response to dilution rate of two microorganisms competing for the same limiting nutrient.

Materials and methods

Organisms and media

Azospirillum brasilense cd (ATCC 29710) and *Arthrobacter giacomelloi*[1] were grown at 30°C in MB_7 medium[2] containing disodium succinate as carbon source. Succinate-sufficient medium contained 5 g/l and succinate-limited medium, 0.75 g/l of the disodium salt. The pH was about 7.0.

Binary continuous culture

Binary continuous culture was established on the basis of the model proposed by Kemp *et al.*[11]. Single cultures of *Azo.brasilense* and *Arth. giacomelloi* were grown continuously in chemostats with working volumes of 100 ml. The media were inoculated with 10 ml of actively growing nitrogen-fixing cultures. Media were supplied by a LKB 2132 MicroPerpex Pump equipped with a two-channel pump head. The overflows from the two vessels were led either to the waste reservoirs or were interconnected and led to a third similar chemostat that functioned as the mixing chamber. The pure continuous cultures were used to inoculate the medium in the mixed culture chemostat once; they were not fed in continuously throughout the experiment. A single channel LKB 2132 MicroPerpex Pump was used to regulate the flow of fresh medium to the mixing chamber. The temperature of the three vessels was controlled at 30°C. The cultures were stirred magnetically and were flushed with a mixture of 99% N_2 and 1% O_2 at a flow rate of 100 ml/min. The following dilution rates (D) per hour were studied: 0.02; 0.035; 0.05; 0.075 (Fig. 1).

Culture viability

The percentage viability of the bacteria in chemostat cultures at various dilution rates was determined by a modified slide-culture technique[6]. *Azospirillum brasilense,* in pure and mixed culture, was inoculated on Difco Phenol Red Dextrose agar containing ($g.l^{-1}$): proteose peptone, 10; dextrose, 5; NaCl, 5; phenol red, 0.018. The different microorganisms were clearly differentiated on this medium as *Azo. brasilense* colonies were pinkish while *Arth. giacomelloi* ones were whitish-cream[4]. In addition *Arth. giacomelloi* in pure culture was inoculated on YG agar medium containing ($g.l^{-1}$): glucose, 10, and yeast extract, 7,[16]. The slides were incubated in a moist atmosphere at 30°C and examined by phase-contrast after 24 h. Each single cell was counted as a dead unit while each microcolony as a viable one: 100 to 200 units were counted and the percentage viability was calculated from the ratio of viable and dead cells.

Saturation constant and maximum specific growth rate

The saturation constant K_s was calculated from the equation[14]:

$$K_s = \frac{\bar{s}(\mu_{max}) - D}{D}$$

where $\bar{s}$ is the steady-state succinate concentration in the culture vessel at a given dilution rate (D) and μ_{max} is the maximum specific growth rate of the bacterium. To determine μ_{max} the

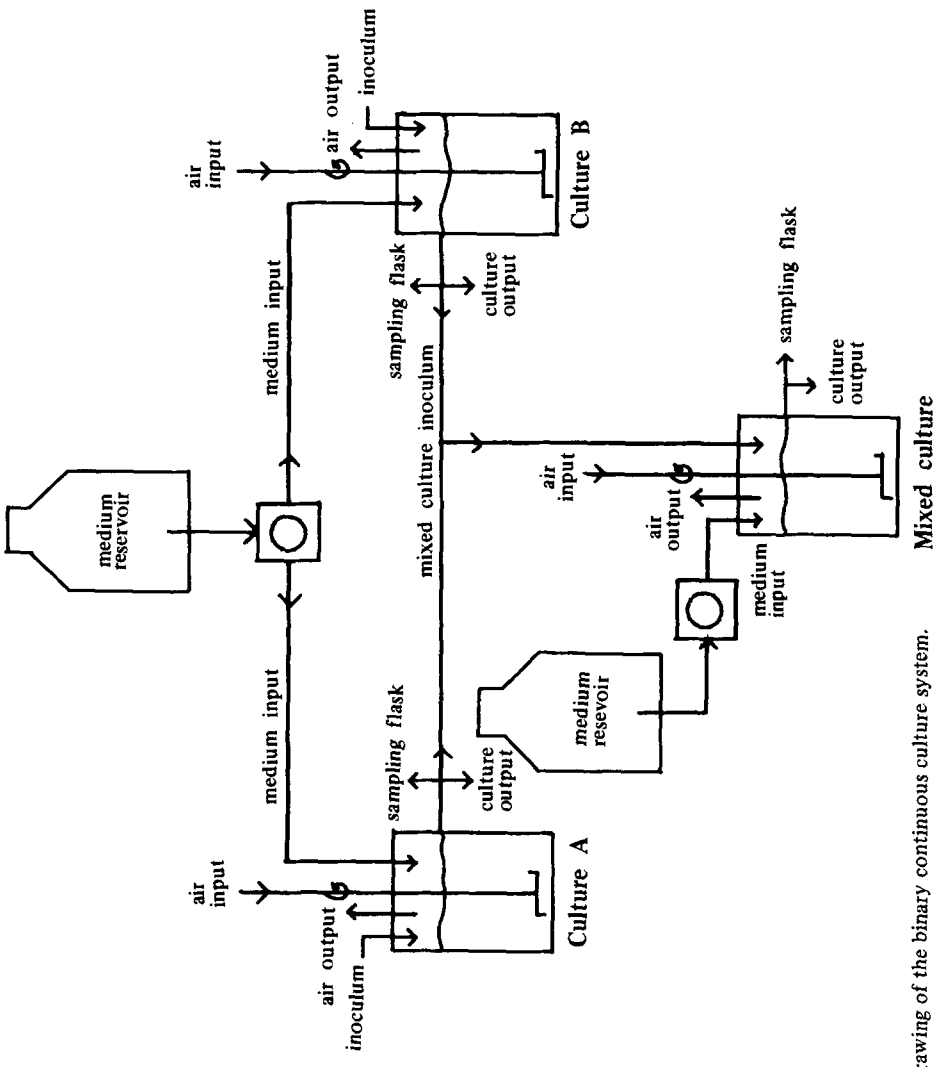

Fig. 1. Schematic drawing of the binary continuous culture system.

bacteria were grown in 500 ml succinate-sufficient batch cultures with a stirring rate of 200 rev.min^{-1}. The cultures were flushed with a gas mixture of 99% N_2 and 1% O_2 at a flow rate of 0.5 l.min^{-1}; Optical densities (O,D.) were recorded continuously and the relationship of O.D. to dry weight of bacteria was calculated on the basis of previously obtained calibration curves. To determine $\bar{s}$, steady-state samples from the cultures grown at D of 0.05 h^{-1} were centrifuged at 16 000 *g* for 15 min and the supernatant liquids analysed for succinate concentration by the method of Williamson[22].

Starvation

Steady-state samples from both single and mixed cultures were aseptically centrifuged (16 000 g, 15 min), washed three times and suspended in 0.033 mol/l phosphate buffer, pH 7.0, and subjected to total nutrient starvation for 14 days at 30°C. During this period several samples were analysed for counts of viable cells, as described previously. The log of the number of cells was plotted against time and subjected to linear regression analysis. The time for the initial viable population to become non-viable (ST_{50}) was calculated from the slope (m) of the regression line of viable cell numbers where $ST_{50} = \ln 2/m$[15].

Rate of oxygen uptake

The rate of oxygen uptake of cultures was measured in an oxygen electrode cell by a Beckman Model 0260 Oxygen Analyzer coupled to a Leeds and Northrup XL 681 Speedomax, 0 to 100 mV multirange potentiometric recorder having a response time of 0.5 sec. The respiration vessel was designed to be completely filled with 9 ml of cell suspension and was tightly sealed by the electrode itself. A water-jacket ensured that the samples, stirred magnetically, could be maintained at 30°C. The recorder was calibrated from zero oxygen to air saturation. Chemostat culture samples were rapidly transferred to the respiration vessel and the ambient O_2 concentration was raised to *ca* 76 μmol/l by bubbling air through the sample for 15 sec.

Analyses

Nitrogenase activity of cultures was determined as previously described[2]. The samples were incubated in the presence of acetylene at $pO_2 = 0.01$ atm. Protein content was measured by the method of Lowry *et al.*[13], using bovine serum albumin as standard. Bacterial dry weight at the steady-states was determined in duplicate by centrifuging and washing 10 ml culture samples and drying the cells to constant weight at 95°C.

Results

Characteristics of growth of Azospirillum brasilense *and* Arthrobacter giacomelloi *in single and mixed cultures*

Azospirillum brasilense and *Arth. giacomelloi* were grown in continuous culture under nitrogen-fixing conditions and succinate limitation at a pO_2 of 0.01 atm. This partial oxygen pressure supported the growth of both organisms: μ_{max} values on succinate were 0.089 h^{-1} for *Azo. brasilense* and 0.076 h^{-1} for *Arth. giacomelloi.* Nevertheless, a pO_2 of 0.01 atm was not optimal either for *Azo. brasilense* or for *Arth. giacomelloi*, as in batch culture under nitrogen-fixing and succinate-sufficient conditions maximum growth for the azospirillum was obtained at pO_2 of 0.006 atm and for the arthrobacter at pO_2 of 0.05 atm. K_s values at D of 0.05 h^{-1} were calculated to be 12.9 μg.ml^{-1} for *Azo. brasilense* (residual succinate 16.6 μg.ml^{-1}) and 13.4 μg.ml^{-1} for *Arth. giacomelloi* (residual succinate 25.8 μg.ml^{-1}).

Table 1. Effect of dilution rate on molar growth yields of *Arthrobacter giacomelloi* and *Azospirillum brasilense* in single and mixed continuous cultures

D (h^{-1})	Y (g dry weight/moles succinate consumed)		
	Arthrobacter giacomelloi	*Azospirillum brasilense*	Mixed culture
0.02	111	72.5	48
0.035	102.5	140	58
0.05	82	130	58
0.075	68	82	60

Table 2. Viability of *Arthrobacter giacomelloi* and *Azospirillum brasilense* in single and mixed continuous cultures

D (h^{-1})	% of viable cells		
	Arthrobacter giacomelloi	*Azospirillum brasilense*	Mixed culture
0.02	93.3	73.6	n.d.
0.035	86	94	93
0.05	87	95	79.3
0.075	83	97	77

When single continuous cultures grown at the same dilution rate achieved steady-states, equal volumes of the azospirillum and the arthrobacter were inoculated into the mixing chamber simultaneously. This third chemostat then functioned as a single-stage continuous culture growing at the same dilution rate. Steady-states in both single and mixed cultures were always reached at all dilution rates. As described[12] *Azo. brasilense* exhibited wall growth.

Table 1 reports the values of molar growth yields (g dry weight/mol of succinate consumed) in both single and mixed cultures. Molar growth yields of *Arth. giacomelloi,* as for other arthrobacters[3,7], were higher at lower dilution rates and decreased with increasing dilution rate. In the azospirillum, instead, molar growth yields were higher at D of 0.035 h^{-1} and 0.05 h^{-1}. The behaviour of this bacterium appeared in agreement with the results obtained in malate-limited cultures of azospirillum by Kloss *et al.*[12]. Molar growth yields of mixed cultures were lower than those of single cultures.

Viability and survival during nutrient starvation

Viability of *Arth. giacomelloi* steady-state cultures was inversely related to growth rate while the azospirillum was directly related. Slightly lower values of viability were obtained in mixed cultures (Table 2).

From the results reported in Table 3 it appears that a constant ratio

Table 3. Effect of dilution rate on ratio of viable number of *Arthrobacter giacomelloi* and *Azospirillum brasilense* in mixed continuous cultures

D (h^{-1})	% of viable cells	
	Arthrobacter giacomelloi	*Azospirillum brasilense*
0.02	43	57
0.035	38.1	61.9
0.05	37.8	62.2
0.075	17.7	82.3

of the two organisms was established in the mixed culture. *Azospirillum brasilense* predominated and constituted about 60% of the total population, but did not outgrow *Arth. giacomelloi.* The value of 82% at D of $0.075\,h^{-1}$, a growth rate very close to that of the arthrobacter μ_{max}, was probably due to a near wash-out of this bacterium.

Samples from both single and mixed cultures were subjected to total nutrient starvation and sampled for survival at different time intervals. The results obtained were subjected to linear regression analysis and the log of the number of viable cells disclosed significant linear relationships with time in both single and mixed cultures (Fig. 2). All regression analyses were significant at $P < 0.01$. On the basis of these analyses 50% survival time values were calculated (Table 4). The results show that survival of the arthrobacter grown in single culture decreased with increasing dilution rate, while in the azospirillum cultures it appeared to be directly related to dilution rate. In mixed culture , viability of the arthrobacter declined less rapidly than in single culture at D of $0.035\,h^{-1}$ and $0.05\,h^{-1}$ while remaining unchanged at D of $0.02\,h^{-1}$. In *Azo. brasilense* the cells grown at D of $0.035\,h^{-1}$ exhibited a more rapid decline in viability than those in single culture at the same dilution rate while the rate of survival at the other dilution rates did not change.

Acetylene reduction activity and respiration rate

Acetylene reduction activity in all succinate-limited continuous cultures were generally very low (Table 5). In batch cultures at the same oxygen pressure under succinate-sufficient conditions nitrogenase activity of *Azo. brasilense* was about 3450 nmoles. mg^{-1} protein. h^{-1} and that of *Arth. giacomelloi* about 70 nmoles. mg^{-1} protein. h^{-1}. The low values of nitrogenase activity in succinate-limited cultures could be due to the scarcity of carbon substrate in the incubation tube, as reported by Kloss *et al.*[12]. An increased sensitivity to oxygen by carbon-limited cultures, as described for *Azotobacter*[5], might also account for the low activities observed.

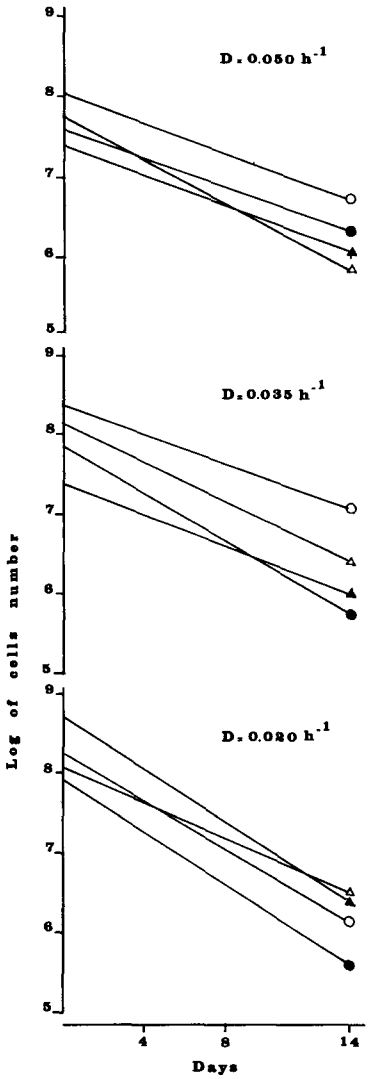

Fig. 2. Linear regression of cell number against time during starvation of steady-state succinate-limited chemostat cultures of *Azo. brasilense* (○, ●) and *Arth. giacomelloi* (△, ▲) in single (open symbols) and mixed (closed symbols) cultures at various dilution rates.

Respiration rates (Table 5) of *Azo. brasilense* and *Arth. giacomelloi* were quite high and showed a direct relationship with growth rate, as could be expected[8]. The values of oxygen uptake rate of mixed cultures were slightly higher than those found in single cultures.

Table 4. Rates of change in viable numbers of *Arthrobacter giacomelloi* and *Azospirillum brasilense* during starvation

D (h^{-1})	*Arthrobacter giacomelloi*				*Azospirillum brasilense*			
	Single culture		Mixed culture		Single culture		Mixed culture	
	Rate[a]	ST_{50}[b] (d)	Rate	ST_{50} (d)	Rate	ST_{50} (d)	Rate	ST_{50} (d)
0.02	−0.108	6.4	−0.117	5.9	−0.144	4.8	−0.162	4.3
0.035	−0.130	5.3	−0.095	7.3	−0.097	7.1	−0.144	4.8
0.05	−0.128	5.4	−0.096	7.2	−0.087	7.9	−0.093	7.45

[a]Slope of regression line of viable cell numbers
[b]50% survival time

Table 5. Acetylene reduction activity and oxygen uptake rate in *Arthrobacter giacomelloi* and *Azospirillum brasilense* in single and mixed continuous cultures

D (h^{-1})	Acetylene reduction activity (n moles/mg protein/h)			Oxygen uptake rate (µl oxygen/mg d w/h)		
	Arthrobacter giacomelloi	*Azospirillum brasilense*	Mixed culture	*Arthrobacter giacomelloi*	*Azospirillum brasilense*	Mixed culture
0.02	2	184	95	60	60	93
0.035	26.8	113	n.d	68	31	89
0.05	n.d	69	n.d	82	102	122
0.075	10	63	76	167	222	n.d

Discussion

Chemostat theory predicts that, unless microbial interactions occur, a single bacterial population will competitively displace all others if a culture is inoculated with a mixture of organisms[20]. Matin and Valdkamp[14] investigated the physiological reasons for the domination of a *Spirillum* sp. at low growth rates in a lactate-limited medium over a *Pseudomonas* sp. which, in its turn, outgrew the *Spirillum* sp. at high growth rates. The four-fold lower K_s values and two-fold lower μ_{max} of *Spirillum* sp. gave it a selective advantage at low substrate concentrations and low growth rates. In the study reported here μ_{max} and K_s values of *Azo. brasilense* and *Arth. giacomelloi* did not differ substantially and syntrophic growth rather than competition seemed to occur in succinate-limited continuous culture.

Molar growth yields and number of viable cells of mixed cultures were much lower than those obtained in pure cultures. Nevertheless, a stable coexistence of the two organisms was maintained at all dilution rates. The bacteria in mixed culture could survive total starvation and were able to increase their respiratory capacity, confirming the potential ability of oligotrophic bacteria to function under restricted nutrient availability conditions[18]. Moreover, mixed culture results have shown

that *Arth. giacomelloi* and *Azo. brasilense* cd are both successful in competition for succinate under nitrogen-fixing conditions.

Although care must be taken in extrapolating these results to field conditions, binary continuous culture showed itself to be a useful tool for helping to understand some of the factors controlling rhizosphere populations.

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