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Trophic Interactions in Soils as They Affect Energy and Nutrient Dynamics. II. Physiological Responses of Selected Rhizosphere Bacteria

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Abstract. Comparative microbial functions in the plant root zone were studied by evaluating rhizosphere-derived *Pseudomonas* and *Arthrobacter* growth in chemostat culture and responses to root-exudate-related nutrients after varied starvation periods. These organisms were chosen to represent zymogenous and autochthonous microbes, respectively. In chemostat culture, the *Pseudomonas* isolate showed increased energy charge and decreased populations with higher growth rates, whereas the *Arthrobacter* had lower energy charge and cell population values which did not change appreciably with growth rate. The responses of these two types of organisms also differed with starvation. The *Pseudomonas* lost its ability to respire efficiently in the presence of several known root exudate components, whereas the *Arthrobacter* isolate, in comparison, maintained a lower but more consistent ability to utilize these nutrients with increased starvation. The *Arthrobacter* also showed increased utilization of several substrates after starvation, suggesting its potential ability to function under restricted nutrient availability conditions. These results suggest that *Pseudomonas*-type organisms in the rhizosphere may best function in periods of more intense exudate release, whereas organisms of the *Arthrobacter*-type may be more efficient at nutrient utilization during periods of lesser nutrient flux. Based on these data the rhizosphere-derived *Pseudomonas* isolate was considered to be an appropriate bacterium to use in more complex rhizosphere microcosm experiments where nutrient flux dynamics would be emphasized.

Introduction

Arthrobacter and *Pseudomonas* organisms have been used in many studies to evaluate the responses of autochthonous and zymogenous groups, respectively, in soils and other environments [10,18,28]. In addition to these studies, more specific *Pseudomonas-Arthrobacter* interactions have been evaluated. Chan and Katznelson [3] showed that *Arthrobacter globiformis*, growing in root extract materials, was inhibited by the presence of a pseudomonad. Pseudomonads from

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an estuarine water were shown to inhibit and agglutinate *Arthrobacter*, and there was an inverse relationship between the numbers of *Arthrobacter* and *Pseudomonas* in the natural environment [24]. Labeda et al. [15] have shown that *Arthrobacter* and *Pseudomonas* populations may achieve maximum levels in sterilized soils independent of the other organism's presence. Physiological studies have also shown that these two groups have marked differences. Thus, pseudomonads are much less resistant to drying [22]. In a general review, Boylen and Ensign [2] indicated that *Arthrobacter* sp. had a 50% survival time of 1680 hr, whereas for *Pseudomonas aeruginosa*, the comparative value was 68 hr.

In this study, the influence of growth rate and starvation effects on utilization of root-exudate-related nutrients was evaluated to allow characterization of organisms to be used in more complex rhizosphere microcosm environments [7], and to allow the further development of bacterial predation models [13] in which main emphasis to date has been placed on the predation of actively growing bacterial cultures.

Materials and Methods

Microorganisms and Culture Conditions

The organisms selected for this study were isolated from the Pawnee Site, intensive study site and field research facility of the Natural Resource Ecology Laboratory, Colorado State University, located on the USDA Science and Education Administration Central Plains Experimental Range in northeastern Colorado. The *Pseudomonas cepacia* and *Arthrobacter* cultures were isolated from the roots of blue grama [*Bouteloua gracilis* H.B.K. (Lag.) ex Steud.] using the root washing technique of Louw and Webley [16] followed by the culturing of organisms on nutrient agar (Difco, Detroit, Michigan).

A standard reference soil solution (RSSB) was used in all studies with the *P. cepacia* and *Arthrobacter* (Drs. C. V. Cole, and D. C. Coleman):

			Compound	*(w/v)%*
RSSC	RSSB	RSS	$CaCO_3$	0.125
			$MgSO_4 \cdot 7H_2O$	0.099
			$CaSO_4 \cdot 2H_2O$	0.98
			$NaHCO_3$	0.008
			K_2SO_4	0.052
			KH_2PO_4	0.068
			Asparagine	0.10
			Glucose	0.20
			$FeCl_2$	0.002
			Biotin	0.001
			Pantothenic acid	0.001
			Thiamine	0.001

The final pH was approximately 7.1. The medium was filtered to remove insoluble materials and was then autoclaved. The medium had a C/N ratio of approximately 5.5.

For use in certain experiments, the glucose and asparagine were omitted and the remaining components used as a reference soil solution (RSS).

Starvation Experiments

Both the *P. cepacia* and *Arthrobacter* were grown separately in 75-ml volumes of the RSSB medium using a 0.75-ml inoculum taken directly from a 24-hr culture. After completion of growth, the cells

were washed by two centrifugations at 8000 × *g* for 15 min, and allowed to starve in the RSS medium for 1-, 7-, and 14-day periods at 26°C, with the cultures shaken on a Lab-Line Gyrotory Shaker operating at 150 rotations per minute.

Chemostat Cultures

The microorganisms used in this study were grown in a 150-ml volume chemostat at 26°C. For nonstarvation experiments the complete medium (RSSC) was used. The cultures were mixed by a magnetic stirring device and aerated at 200 ml/min for *P. cepacia* and at 35 ml/min for the *Arthrobacter* culture. With higher aeration rates the *Arthrobacter* culture was subject to washing out of the chemostat vessel.

The chemostats were inoculated from slant cultures and dilution rates (D) of 0.1, 0.2, 0.3, 0.4, and 0.5 were used for the *Arthrobacter*, and the same rates, plus 0.6 and 0.7, were used for the *Pseudomonas* test system. At each dilution rate a minimum of five complete volumes were passed through the growth chamber before sampling began to assure steady-state conditions. The dilution rates were chosen to reflect differing calculated maximum specific growth rates for the two organisms, being 0.51 hr^{-1} for the *Arthrobacter*, and 0.69 hr^{-1} for the *Pseudomonas*.

Oxygen Use Measurements

A Yellow Springs Instrument Company Biological Oxygen Monitor and a Clark polarographic electrode were used to measure respiration after the addition of root exudates and samples of known root exudate components. A Gilford microsyringe was used to inject 0.05 ml of the carbon source (1.0 mM) into 3.0 ml of cell suspension. Results are expressed as the difference between the basal oxygen utilization rate and the rate after carbon source addition. Sterile blue grama root exudates were supplied by Dr. U. G. Bokhari, Natural Resource Ecology Laboratory, Colorado State University.

Energy Charge Measurements

The energy charge was calculated to provide information on the acceptability of these organisms as food sources for predators in later modeling studies, and as an indicator of the possible responses of each organism to starvation stress in relation to growth rate. This analysis was carried out using an American Instrument Company Photomultiplier microphotometer, a Houston Instruments Omniscribe integrating strip chart, and a repeating dispenser which allowed automatic delivery of 0.1 ml of the prepared luciferin-luciferase enzyme. An integrated value for the total light emitted for the first 30 seconds after injection was used in all calculations, using a 0.45-ml sample of a particular extract.

Extraction Procedure

The extraction procedure of Lundin and Thore [17] was used. First, 2 ml of bacterial culture was placed in 1.2 ml of 0.51 M trichloroacetic acid (TCA) and 17 mM ethylenediaminetetraacetic acid (EDTA), mixed well, and allowed to stand at 0°C for 15 min. The TCA was extracted by three washings with 4-ml volumes of water-saturated diethylether. The first phase separation was facilitated by a short centrifugation (10,000 × *g* for 10 min). After the final ether extraction, water-saturated nitrogen gas was bubbled through the water phase for 5 to 10 min to remove solubilized ether. The sample was diluted to contain 10% bacterial culture and 2 mM EDTA in a Tris (hydroxymethyl) aminomethanesulfonic acid buffer, pH 7.75, containing 20 mM Tris and 2 mM EDTA. The samples were then frozen at −25°C until assayed.

The method for luciferase assay was essentially that recommended by Pradet [21]. For the determination of adenosine triphosphate (ATP), 1.8 ml of the cell extract was added to 0.45 ml of a 75-mM potassium phosphate buffer, pH 7.3, 15 mM in $MgCl_2$.

For ATP and adenosine diphosphate (ADP) determinations, 1.8 ml of the cell extract was added

to 0.45 ml of a solution containing 75 mM potassium phosphate (pH 7.3), 15 mM $MgCl_2$, 0.5 mM phosphoenolpyruvate, and 20 μg of pyruvate kinase (the last two from Sigma Chemical Company).

The total adenylate pool was determined by mixing 1.8 ml of cell extract with 0.45 ml of a solution containing 75 mM potassium phosphate (pH 7.3), 15 mM $MgCl_2$, 0.5 mM phosphoenolpyruvate, 20 μg of pyruvate kinase, and 25 μg of adenylate kinase (the last three from Sigma Chemical Company). The adenylate kinase was dialyzed against 50 mM potassium phosphate, pH 7.3, for 24 hr at 4°C before use.

The three mixtures were incubated at 30°C for 15 min and then held at 0°C until assayed. ADP and adenosine monophosphate (AMP) were determined by difference.

The instrument was routinely standardized against an ATP solution (Sigma) using an appropriate concentration range.

One vial of the crystalline luciferin-luciferase mixture (DuPont Instruments Company) was dissolved in 3 ml of the buffer provided. At this concentration the enzyme mixture was stable for 4 days at 0° to 4°C.

Enumeration Procedures

Total cell numbers were determined using a Petroff-Hauser bacterial chamber.

Determinations of microbial viability were performed using the microslide culture technique [20] and spread plates. The microslide cultures were prepared by the method of Ensign and Wolfe [9] with several modifications. Peptone (1.0% w/v)-yeast extract (0.1% w/v), solidified with 1.5% (w/v) Difco purified agar, was used as a growth medium. While hot, 4 ml of this medium was added to a sterile Petri dish and spread to obtain a very thin, flat agar surface. The culture was spread on top of this agar surface and incubated for 4 to 6 hr at 25°C. At the end of the incubation, a 1-cm square was cut out of the agar and placed on a slide and observed under phase-contrast microscopy for evidence of cell division initiation.

Results

The responses of the *Arthrobacter* isolate to the varying dilution rates in chemostat culture are shown in Fig. 1. With increasing dilution rate, the energy charge remained relatively constant, and a slight increase in the total viable cell count was observed. Based on the microscopic total cell number assay, with increasing dilution rate, an increase in the percentage of viable cells in the population occurred, and cell dry weights in the range of 1 to 2 mg/ml were maintained in the chemostat over the range of dilutions tested.

In contrast, the *Pseudomonas* culture showed more distinct responses with

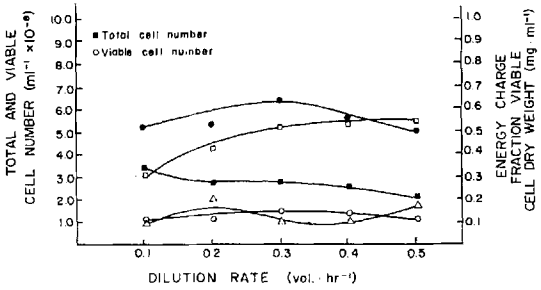

Fig. 1. *Arthrobacter* energy charge and population parameter responses at various dilution rates (● energy charge, □ = % viable cells, Δ = cell dry weight).

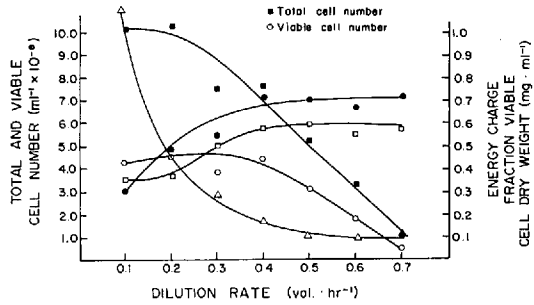

Fig. 2. *Pseudomonas* energy charge and population parameter responses at various dilution rates (• = energy charge, □ = % viable cells, Δ = cell dry weight).

varying growth rates (Fig. 2). The energy charge increased above a dilution rate of approximately 0.2, and from a dilution rate of 0.4 to 0.7 the energy charge remained near 0.7. As with the *Arthrobacter* culture, with increasing dilution rate, an increased percentage viability was noted. A point of contrast between this organism and the *Arthrobacter* isolate was its tendency to maintain lower populations in the chemostat at higher growth rates. This decrease appeared to be linear with increased dilution rates, and the percent viability tended to be lower at the 0.1 to 0.2 dilution rates, similar to the responses noted for energy charge over the range of growth rates which were tested. The cell dry weight data also decreased with higher dilution rates, although this appeared to be an exponential type change, in contrast with the total cell count which exhibited a more linear relationship.

The energy charge responses for the two types of organisms with increasing starvation are shown in Table 1. Both organisms showed decreased energy charge values after starvation. At the end of the starvation time, no differences in energy charge (0.44 and 0.44) were observed, and the *Pseudomonas* culture was 18% to 19% viable, although the *Arthrobacter*, in contrast, exhibited 70% to 80% viability by the microculture procedure used in the study.

In contrast to the relatively constant energy charge values which were observed in relation to starvation time, major changes in the ability to utilize root-exudate-related substrates were noted for the two organisms (Tables 2 and 3). Generally the *Pseudomonas* culture showed higher initial oxygen uptake rates for the substrates which could be utilized, but this organism appeared to show a greater relative loss of utilization capability during starvation, although the absolute values for utilized substrates were generally higher than for the *Arthrobacter* at the end of the 14-day period. The *Pseudomonas* culture showed

Table 1. Energy charge of *Pseudomonas* and *Arthrobacter* cultures after starvation

	Starvation Time (days)		
Organism	1	7	14
Pseudomonas	0.82 ± 0.07	0.39 ± 0.07	0.44 ± 0.03
Arthrobacter	0.51 ± 0.02	0.45 ± 0.08	0.44 ± 0.06

Table 2. Root exudate effects on *Pseudomonas* respiration (μl O_2 · min^{-1} · mg^{-1}) after various starvation times[a]

Substrate class	Substrate	Starvation Time (days)		
		1	7	14
Root exudate		4.32 ± 0.9	0.49 ± 0.03	0.17 ± 0.02
Organic acids	Fumaric	—[b]	—	—
	Malic	—	—	—
	Oxalic	0.17 ± 0.3	0.15 ± 0.4	0.07 ± 0.01
	Succinic	—	—	—
Amino acids	Alanine	0.09 ± 0.02	—	—
	Aspartic	1.88 ± 0.43	0.23 ± 0.08	0.14 ± 0.03
	Glutamic	2.87 ± 0.17	0.45 ± 0.32	—
	Histidine	0.29 ± 0.02	0.06 ± 0.01	0.07 ± 0.03
	Lysine	—	—	—
	Methionine	—	—	—
	Proline	—	—	—
	Serine	—	—	—
Sugars	Fructose	0.97 ± 0.15	0.45 ± 0.07	0.29 ± 0.01
	Glucose	6.67 ± 1.03	0.14 ± 0.01	0.04 ± 0.01

[a] No responses were shown with asparagine, glycine, or leucine.
[b] No change noted.

Table 3. Root exudate effects on *Arthrobacter* respiration (μl O_2 · min^{-1} · mg^{-1}) after various starvation times[a]

Substrate Class	Substrate	Starvation Time (days)		
		1	7	14
Root exudate		0.30 ± 0.07	0.08 ± 0.01	0.10 ± 0.02
Organic acids	Formic	0.15 ± 0.06	—[b]	—
	Malic	0.58 ± 0.11	—	—
	Oxalic	0.58 ± 0.03	0.04 ± 0.01	0.03 ± 0.00
	Succinic	—	—	—
Amino acids	Alanine	0.59 ± 0.06	0.04 ± 0.01	0.03 ± 0.02
	Aspartic	0.59 ± 0.12	0.04 ± 0.00	0.03 ± 0.01
	Glutamic	—	0.01 ± 0.00	0.02 ± 0.00
	Histidine	0.29 ± 0.07	0.04 ± 0.01	0.03 ± 0.02
	Lysine	—	0.02 ± 0.00	0.02 ± 0.01
	Methionine	—	—	—
	Proline	0.59 ± 0.09	0.03 ± 0.01	0.02 ± 0.00
	Serine	—	—	—
Sugars	Fructose	—	0.14 ± 0.03	0.12 ± 0.04
	Glucose	0.30 ± 0.93	0.24 ± 0.08	0.08 ± 0.01

[a] No responses were shown with asparagine, glycine, and leucine.
[b] No change noted.

a narrower range of utilizable substrates (8 of 18), as compared with the *Arthrobacter* culture which utilized 12 of the 18 substrates. Three substrates (glutamate, lysine, and fructose) were utilized only after the initial assay time.

Both organisms showed an ability to utilize the components in the sterile root-exudate preparation, and the *Pseudomonas* culture utilized fewer of the organic acids and amino acids tested. However, both organisms were able to utilize the two sugars which were tested, fructose and glucose. After 14 days of starvation, fructose was used at the highest rate for the *Pseudomonas* culture, whereas for the *Arthrobacter* culture, glucose was the substrate which stimulated the highest oxygen utilization rate. These results suggest that after starvation, carbohydrate-derived substrates would be utilized efficiently by both microorganisms.

Discussion

The study has shown that two organisms considered to be representative of the zymogenous and autochthonous components of the soil microflora, as originally noted by Winogradsky [28], show different physiological responses at varied growth rates, in their viability retention upon starvation, and in their ability to utilize nutrients considered to be components of plant root exudates.

The differences in energy charge with variation in growth rate observed for these two organisms are also noted in the available literature. In most cases the energy charge has been found to be relatively constant in spite of varied microbial growth rates. Holms et al. [12] noted a constant ATP level in *Escherichia coli* with varied growth rates, and Dolezal [8] noted with *Citrobacter* that the energy charge remained relatively constant over a range of dilution rates from 0.03 to 0.29. In contrast, Ho and Munk [11] found that the energy charge of a *Candida utilis* grown in chemostat culture decreased with increasing dilution rate until energy uncoupling occurred, and an increase in the energy charge and ATP level was observed. The clear differences in energy charge status noted between the *Arthrobacter* and *Pseudomonas* cultures used in this study also suggest that there may be major differences in metabolic regulation between these two types of organisms. The decrease in *Pseudomonas* populations which occurred at higher dilution rates does not appear to be related to oxygen limitation, as an oxygen level of between 2 and 3 mg/liter was observed at all dilution rates. This was caused by the tendency of this organism to be removed from the culture vessel during aeration, which appeared to increase at higher dilution rates.

The decreased viability percentages observed with these two organisms at lower dilution rates have also been noted in the previous literature, where similar carbon-limited growth conditions were used [19,26]. These conditions were chosen to represent the growth environment with plant-root exudates, based on the recommendation of Stouthamer [25] that the determination of growth parameters "must preferably be performed with substrate-limited cultures."

The decreased viability of *Pseudomonas* after starvation, in comparison with the responses of *Arthrobacter,* has also been noted by Boylen and Ensign [2] in an earlier study. In spite of this decreased viability, the energy charge of the two populations remained essentially constant. For *E. coli,* Walker-Simmons and

Atkinson [27] found that growing cells maintain an energy charge of at least 0.8 and that viability may be maintained at values of between 0.8 and 0.5. As the energy charge values remained relatively constant for the two organisms (0.4 to 0.5) during starvation, in spite of markedly different viability values, energy charge, as an isolated parameter, may not be suitable for predicting viability or survival in low-nutrient environments.

However, with information on the absolute adenylate levels, biomass, and percent viability of microbial populations after starvation, it may be possible to better utilize energy charge information, especially in relation to the evaluation of stresses imposed in natural environments. In this study, it was noted that the *Pseudomonas* culture had decreased energy charge values after washing in the laboratory, which was not shown by the *Arthrobacter*. Organisms can rapidly lose their adenine nucleotide pools [4,14,15], which could reflect differing sensitivities to physical handling or variations in environmental conditions. Based on our studies, the *Arthrobacter* would appear to be better able to withstand such stresses.

The responses of the two organisms to the presence of sterile root exudate and to compounds known to be present in root exudate also emphasized the differing physiological capabilities of these organisms. Under conditions of active plant growth or stress, with exudates being released at greater rates, the *Pseudomonas* type cultures would be able to utilize these nutrients much more efficiently and could be assumed to achieve functional predominance in the rhizosphere, as postulated by Chan and Katznelson [3]. The increased ability to utilize carbohydrates and amino acid-related nutrients also suggests the ability of both these microbial types to utilize the major components which might be released from plant roots [23].

These studies suggest that in developing microcosms for further analysis of microbial functions in the rhizosphere environment [1,5,6], if active plant growth and root exudate release are assumed, organisms of the *Pseudomonas* type would provide an appropriate primary microbial consumer in such systems.

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