

J. A. OCAMPO, J. M. BAREA, E. MONTOYA*

AMENSALISM AS AN ECOLOGICAL FACTOR AFFECTING
THE ESTABLISHMENT OF AZOTOBACTER
AND "PHOSPHOBACTERIA" IN THE RHIZOSPHERE

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Several antagonistic ecological factors contribute to the reestablishment of the natural equilibrium in the rhizosphere when Azotobacter, phosphobacteria, or other microorganisms are massively introduced into the root zone. Amensalism is one such factor and its effect on the establishment of the above mentioned bacteria in the rhizosphere is tested in this paper. Amensalistic microorganisms acting as antibiotic producers during the early stages of growth may influence the colonization of plant roots, while lytic enzymes later antagonized the establishment of Azotobacter or phosphobacteria in the root region.

Ocampo *et al.* [10] found that there was a protooperative interaction [15] between Azotobacter and phosphate-solubilizing bacteria when inoculated into the plant rhizosphere, however, numbers of Azotobacter and phosphobacteria introduced were not in all cases tested.

Experiments carried out in our laboratory tried to elucidate which of the several ecological factors in the root zone may play a major role in depressing the establishment of these microorganisms. The effects of root extracts, phages, Bdellovibrio, and soil bacteriostasis were tested and discussed [8, 11]. Although each of these factors had an antagonistic influence on the establishment of Azotobacter and phosphobacteria, none of them was decisive on the bacterial decrease in the rhizosphere. Negative interactions [15] between natural microbiota and inocula are probably the major reason for the failure of Azotobacter and phosphobacteria establishment.

* Dr. Juan-Antonio Ocampo, Dr. José-Miguel Barea, Prof. Dr. Enrique Montoya — Microbiology Department, Zaidín Experimental Station, Avda. Cervantes, 1, Granada, Spain.

Amensalism is undoubtedly one of the most important factors of these negative interactions [15]. Although the most usual form of amensalism in soil is that depending on antibiotic production, the ability of some microorganisms to lyse cells of other microbes is also a form of amensalism [15]. Amensalism had often been demonstrated between soil microorganisms and *Azotobacter*, and antibiotic-producers [2, 7, 12, 16] and lytic organisms [4, 13] against *Azotobacter* have been described. However there is little evidence of amensalism towards phosphobacteria [6].

In this paper we have studied the possible role of amensalistic organisms in the establishment of *Azotobacter* and phosphobacteria in the rhizosphere.

MATERIAL AND METHODS

Microorganisms, plants, and treatment used were described by Ocampo *et al.* [10]. Unsterilized field soil No. 1 amended with 2% (w/w) of farmyard manure was used. Lavender (*Lavandula spica* var. *vera* L.) was the test plant. There were four inoculation treatments: C, control (autoclaved bacterial cultures); P, inoculum of phosphate-solubilizing bacteria; A, inoculum of *Azotobacter*; and A+P. There were also four NPK treatments: 1 = no NPK added; 2 = NPK added at inoculation time; 3 = NPK added in the middle of the experiments and 4 = 2+3 treatments. The NPK treatment consisted of 10 mg/Kg soil of a nutrient solution containing 8.8 g KH_2PO_4 , 25.70 g $\text{Ca}(\text{NO}_3)_2$, and 12.0 g $\text{NH}_4(\text{NO}_3)$ per litre of distilled water. The inocula, seedlings, pots, etc. were prepared as described by Ocampo *et al.* [10]. Numbers of *Azotobacter* and phosphobacteria were also recorded as described before [10].

AMENSALISM TOWARDS GROWING CELLS OF AZOTOBACTER AND PHOSPHOBACTERIA

The amensalism towards inoculated bacteria (*Azotobacter* and phosphobacteria) was tested in samples taken from the rhizosphere at 15-day intervals.

The method used was a bilayer technique. Ten ml of L medium [11] inoculated with appropriate suspensions-dilutions of rhizosphere soil was used for the base layer. Petri dishes were incubated at 28°C for 48 h. Plates which contained 50-100 colonies were then covered with semisolid agar containing the test bacteria. For this semisolid second layer 2 ml of either Brown's medium for *Azotobacter* [3], or L medium for phosphobacteria [11] was used. These media contained 0.5% (w/w) agar and were mixed, at 45°C, with 0.3 ml of each one of three *Azotobacter* spp. (*A. chroococcum*, A₁; *A. beijerinckii*, A₂; and *A. vinelandii*, A₃) or of each of three

phosphobacteria spp. (*Bacillus* sp., A₂₄; *Pseudomonas* sp., J₂; and *Agrobacterium* sp., L).

The Petri dishes were kept at 28°C for 48 h. Colonies with a clear zone of inhibition were counted as being due to amensalism towards Azotobacter or phosphobacteria, respectively. These numbers were used as an index.

AMENSALISM TOWARDS RESTING CELLS OF AZOTOBACTER AND PHOSPHOBACTERIA SOIL

For assessing amensalism towards resting cells of Azotobacter and phosphobacteria a technique based on that described by Sing [14] was used. Ten ml 1.5% water agar was used as a base layer; the second layer contained 2 ml of thick suspension in sterile distilled water of four day-old cultures of each of the three Azotobacter spp. (*A. chroococcum*, A₆; *A. beijerinckii*, A₅; and *A. vinelandii*, A₄) or each of the three phosphobacteria (*Bacillus* sp., A₂₄; *Pseudomonas* sp., J₂; and *Agrobacterium* sp., L). Bacterial suspensions were centrifuged and washed three times before spreading.

30 particles of each experimental rhizosphere soil were transferred to the surface of plates, which were then incubated at 28°C for 4 to 6 days. The number of clear lytic zones (of about 12-16 mm in diameter) around the grains were used as an index of lytic activity. This was checked under the microscope.

RESULTS

Table 1 and 2 show counts of Azotobacter and phosphobacteria recovered from the lavender rhizosphere in each experimental soil inoculated with Azotobacter or phosphobacteria alone or both. In all cases, the number of Azotobacter and phosphobacteria declined, but, at the end of the experiment, there were more Azotobacter and phosphobacteria cells in the rhizosphere of plants inoculated with both Azotobacter and phosphobacteria than when Azotobacter and phosphobacteria were inoculated separately.

Tables 3 and 4 show amensalistic activity towards growing Azotobacter and phosphobacteria, respectively. This activity, being greater in A+P treatment, increased in the middle of the experiment, declined later, and then remained stable at the end of the experiment.

Tables 5 and 6 show that the amensalistic effect by lytic microorganisms, expressed as a percentage of soil grains which had this activity, fluctuated throughout the experiment, but was highest at harvest. This activity was irrespective of the treatment (Table 5 and 6).

TABLE 1. NUMBERS OF AZOTOBACTER CELLS IN LAVENDER RHIZOSPHERE

NPK treatment	Inoculation treatment	Nos. ($\times 10^4$)/ dry rhizosphere soil, weeks after seedling inoculation							
		2	4	6	8	10	12	14	16
1	A	440	900	1400	240	132	49	36	30
	A+P	1250	1300	2000	650	500	235	240	190
2	A	340	920	1600	150	170	108	27	26
	A+P	390	980	1900	230	100	95	100	70
3	A	(The same figures as in NPK-treatment 1)					50	27	32
	A+P						330	100	90
4	A	(The same figures as in NPK-treatment 2)					77	60	50
	A+P						300	80	60

Explanation: Data are mean values of 10 replicates; Standard errors never exceeded 10% of any value. 1—no NPK added; 2—NPK added at inoculation; 3—NPK added at the middle of the assay; 4—2+3 treatments. A—Azotobacter-inoculated pots; P—phosphobacteria-inoculated pots.

TABLE 2. NUMBERS OF PHOSPHATE-SOLUBILIZING BACTERIA IN LAVENDER RHIZOSPHERE

NPK treatment	Inoculation treatment	Nos. ($\times 10^5$)/ dry rhizosphere soil, weeks after seedling inoculation							
		2	4	6	8	10	12	14	16
1	C	32	25	14	5	6	5	3	1
	A	40	27	27	8	8	6	5	3
	P	60	81	48	17	17	15	14	12
	A+P	185	366	130	78	60	60	50	46
2	C	25	37	18	5	6	9	12	3
	A	30	90	40	16	12	11	14	7
	P	60	120	60	20	26	30	31	30
	A+P	70	216	140	90	80	70	65	60
3	C	(The same figures as in NPK-treatment 1)					15	12	9
	A						2	18	9
	P						17	28	15
	A+P						90	80	50
4	C	(The same figures as in NPK-treatment 2)					14	14	4
	A						9	19	16
	P						42	30	30
	A+P						110	60	70

Explanation: as in Table 1; C = Control.

DISCUSSION

Amensalistic microorganisms able to antagonize growing cells of Azotobacter and phosphobacteria thus, by means of antibiotic substances, may have some influence on the establishment of these bacteria during

early phases of colonization of the plant. They were very abundant in the rhizosphere and could exclude many inoculated bacteria, but the number of these amensalistic microorganisms decreased and later remained stable. The decrease may be due to the lower number of inoculated bacteria present in the rhizosphere by the end of the assay. In fact, there was at harvest a higher amensalistic activity in the A+P treatments, in which the number of inoculated bacteria remained higher (Tables 1 and 2).

The lysis of resting cells of *Azotobacter* and phosphobacteria might be attributed to the action of lytic enzymes, depending on how the technique was performed. This lytic activity fluctuated throughout the experiment, but increased at harvest. The fluctuations perhaps might be due to inactivation of these enzymes or adsorption to clay minerals and other particulates [15]. But, some of them are active for a longer time [5]. When the total microflora decreased the lytic activity increased.

Although it is difficult to elucidate which ecological factors play the major role in the decline of the establishment of *Azotobacter* and phosphobacteria in the rhizosphere, the evidence presented in this paper in-

TABLE 3. NUMBERS OF MICROORGANISMS AMENSALISTIC TOWARDS GROWING CELLS OF AZOTOBACTER

NPK treatment	Inoculation treatment	Nos. ($\times 10^8$)/g dry rhizosphere soil, weeks after seedling inoculation							
		2	4	6	8	10	12	14	16
1	C	9	25	11	7	5	2	2	1
	A	6	14	20	5	3	5	5	5
	P	9	14	7	5	2	3	2	1
	A+P	13	14	8	14	12	11	9	9
2	C	9	10	11	11	6	4	3	2
	A	2	18	37	33	14	3	4	4
	P	6	9	9	13	6	4	4	3
	A+P	7	38	27	47	21	9	5	5
3	C						2	2	1
	A	(The same figures as in NPK-treatment 1)					3	6	3
	P						2	2	2
	A+P						10	10	9
4	C						2	3	1
	A	(The same figures as in NPK-treatment 2)					13	6	6
	P						10	3	3
	A+P						16	4	4

L.S.D. (5%) = 1.29

NOTE: Each number is the average of 15 replicates (5 for each of the 3 *Azotobacter* tested). Differences between periods rarely differed more than 5%. 1—no NPK added; 2—NPK added at inoculation; 3—NPK added at the middle of the assay; 4—2+3 treatments. C—Control; A—*Azotobacter*-inoculated pots; P—phosphobacteria-inoculated pots. L.S.D. (5%)—Least Significant Difference at P=0.05 for comparison of any two values.

TABLE 4. NUMBERS OF MICROORGANISMS AMENSALISTIC TOWARDS GROWING CELLS OF PHOSPHOBACTERIA

NPK treatment	Inoculation treatment	Nos. ($\times 10^8$)/g dry rhizosphere soil, weeks after seedling inoculation							
		2	4	6	8	10	12	14	16
1	C	8	7	6	3	2	2	1	1
	A	6	13	22	3	3	3	1	1
	P	16	6	12	12	9	8	4	3
	A+P	10	22	11	14	13	13	7	6
2	C	4	10	7	8	6	2	1	1
	A	4	8	18	16	10	5	1	1
	P	3	7	15	15	12	11	3	2
3	A+P	4	52	26	45	21	17	7	4
	C						1	3	1
	A	(The same figures as in NPK-treatment 1)					6	4	3
	P						10	3	2
4	A+P						17	9	6
	C						1	1	1
	A	(The same figures as in NPK-treatment 2)					4	2	2
	P						12	3	2
	A+P						9	4	2

L.S.D. (5%) = 1.20

No 1: Each number is the average of 15 replicates (5 for each of the 3 phosphobacteria tested). Differences between species rarely differed by more than 5%. 1—no NPK added; 2—NPK added at inoculation time; 3—NPK added at the middle of assay; 4—2+3 treatments. C—Control; A—Azotobacter-inoculated pots; P—Phosphobacteria-inoculated pots. L.S.D. (5%)—Least Significant Difference at 5% for comparison of any two values.

TABLE 5. AMENSALISTIC ACTIVITY ON RESTING CELLS OF AZOTOBACTER

NPK treatment	Inoculation treatment	Percentage Nos. of grains with lytic activity, weeks after seedling inoculation							
		2	4	6	8	10	12	14	16
1	C	14	39	34	37	71	47	69	91
	A	25	31	37	33	54	42	74	100
	P	18	22	30	18	52	70	90	95
	A+P	34	42	45	41	54	40	100	100
2	C	37	10	27	18	39	37	83	92
	A	67	31	66	35	59	58	74	96
	P	33	17	71	26	40	34	100	100
3	A+P	11	29	57	44	61	27	94	100
	C						51	79	85
	A	(The same figures as in NPK-treatment 1)					81	97	100
	P						59	96	100
4	A+P						61	85	100
	C						90	80	97
	A	(The same figures as in NPK-treatment 2)					48	89	99
	P						73	62	93
	A+P						70	97	100

L.S.D. (5%) = 9.12

Explanations: as in Table 3.

icates that amensalism by lytic microorganisms is one of the most important in the survival of this inoculated bacteria. Contrary to the antagonistic factors previously found [1, 8, 9, 11], amensalism by lytic activity increased during the assay. Nevertheless, as these precedent papers indicated, the decline of the establishment of inoculated bacteria might not only be due to a particular factor but also to all factors tested that contribute to reestablishment of the natural equilibria in the rhizosphere.

TABLE 6. AMENSALISTIC ACTIVITY ON RESTING CELLS OF PHOSPHOBACTERIA

NPK treatment	Inoculation treatment	Percentage Nos. of grains with lytic activity, weeks after seeding inoculation							
		2	4	6	8	10	12	14	16
1	C	14	63	22	65	92	71	50	80
	A	21	78	71	52	77	72	64	88
	P	26	72	48	29	75	80	100	100
	A+P	26	75	73	60	58	84	58	88
2	C	39	73	47	31	85	76	48	84
	A	17	57	56	22	87	69	72	76
	P	13	46	69	52	82	98	73	99
	A+P	26	76	69	58	91	80	98	100
3	C						83	70	85
	A	(The same figures as in NPK-treatment 1)					78	70	82
	P						77	100	100
	A+P						82	78	98
4	C						88	67	92
	A	(The same figures as in NPK-treatment 2)					75	60	87
	P						61	79	91
	A+P						83	96	100

L.S.D. (5%) = 10.35

Explanations: as in Table 4.

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AMENSALIZM JAKO EKOLOGICZNY CZYNNIK ODDZIAŁUJĄCY
NA UTRZYMYWANIE SIĘ AZOTOBAKTERIA I „FOSFOBAKTERII”
W RIZOSFERZE

Antagonistyczne mikroorganizmy, działające jak organizmy produkujące antybiotyki, w ciągu wcześniejszych faz wzrostu mogą wpływać na powstawanie kolonii wólk korzeni roślin, podczas gdy później, enzymy lityczne przeciwdziałają się utrzymywaniu się Azotobakteria lub fosfobakterii w strefie korzeniowej.

Translator: the authors