Interactions between *Azotobacter* and "phosphobacteria" and their establishment in the rhizosphere as affected by soil fertility[1]

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The effects on plant growth of "bacterial fertilizers" prepared from *Azotobacter* spp. and phosphate-solubilizing bacteria ("phosphobacteria") have been the subject of much controversy. Cases where no plant-growth stimulation occurred may often be accounted for by the failure to establish the bacterial inocula in the rhizosphere.

Three factors that may influence inocula establishment, i.e. soil fertility, manuring, and interactions between *Azotobacter* and "phosphobacteria," were examined in pot experiments, designed for statistical analysis, in two neutral–alkaline soils, using lavender plants (*Lavandula spica* L.). During the experiments the numbers of *Azotobacter* and "phosphobacteria" were counted. Dry weights of roots and shoots were recorded after 16 weeks of growth.

At the end of the experiments there were always more *Azotobacter* and "phosphobacteria" in the rhizospheres when plants were inoculated with both groups of organisms together than when they were inoculated singly. Addition of 2% farmyard manure to the richer soil enhanced this effect.

Plant growth was greatest when seedlings were inoculated with both *Azotobacter* and the "phosphobacteria."

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Les effets sur la croissance des plantes de fertilisants bactériens préparés à partir d'*Azotobacter* spp. et de bactéries solubilisant les phosphates ("phosphobacteria") ont été le sujet de plusieurs controverses. Des cas où il n'y a pas de stimulation de la croissance des plantes peuvent souvent être expliqués par le manque d'établissement des inoculats bactériens dans la rhizosphère.

Nous avons étudié trois facteurs qui peuvent influencer l'établissement des inoculats, i.e. la fertilité du sol, la fumure et les interactions entre *Azotobacter* et "phosphobacteria," dans des expériences en pots désignées pour des analyses statistiques dans deux sols neutre–alcalin, en utilisant des plants de lavandre (*Lavandula spica* L.). Au cours des expériences les nombres d'*Azotobacter* et de phosphobacteria furent comptés. Les poids secs des racines et des tiges furent déterminés après 16 semaines de croissance.

A la fin des expériences il y avait toujours plus d'*Azotobacter* et de "phosphobacteria" dans les rhizosphères où les plantes furent inoculées avec les deux groupes d'organismes ensemble, que lorsque les plantes furent inoculées séparément. L'addition de 2% de fumier de ferme au sol le plus riche accroît cet effet.

La croissance des plantes fut plus grande lorsque les semis furent inoculés avec les deux cultures *Azotobacter* et "phosphobacteria." [Traduit par le journal]

Introduction

Inoculation of plant rhizosphere with microorganisms can temporarily or permanently change the balance of the rhizosphere population. Such changes may sometimes enhance plant growth, depending on the duration of the effect. In this respect bacterial fertilizers prepared from *Azotobacter*, *Rhizobium*, and phosphate-solubilizing bacteria ("phosphobacteria" according to Ramos-Cormenzana 1970) have been used extensively (see reviews by Dommergues and Mangenot 1970; Mishustin and Shil'nikova 1971; and Brown 1974).

When efficient strains of Rhizobium are used as seed inoculants, they usually benefit the plant because they can become well established inside the plant roots by forming nodules which fix nitrogen symbiotically. *Azotobacter* or "phos-

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phobacteria," however, must become established in the rhizosphere in competition with many other microorganisms, thereby disturbing the normal biological balance in the root zone. It has been stated that inoculation can be expected to improve plant growth mainly if the bacteria are established and grow well in the rhizosphere (Brown *et al.* 1964, 1968).

Cooper (1959), after his visit to the Soviet Union, pointed out that the greatest benefits from using bacterial fertilizers were obtained on fertile soils, with high organic matter content and pH close to neutral. Obviously these factors can improve bacterial growth and the establishment of a large population of inoculated microorganisms.

This paper describes how *Azotobacter* and "phosphobacteria," when inoculated into plant rhizosphere in soils of different fertility, with and without manure, could reciprocally influence their establishment in the root region. The effects of such bacterial establishment on plant growth are also reported.

Material and Methods

Microorganisms

Three phosphate-solubilizing bacteria, a *Pseudomonas* sp., an *Agrobacterium* sp., and a *Bacillus* sp. were selected (among 50 isolated) as efficient solubilizers of sodium phytate and rock phosphate, in pure culture (these bacteria produce plant-growth hormones, according to Barea, Navarro, and Montoya, to be published). Inoculum was prepared by growing each bacterium in the medium of Brown (1972) in shake cultures for 10 days at 28°C. At the time of inoculation the three species were combined. This mixed culture contained about 10^9 cells/ml.

Three *Azotobacter* strains, A_6 (*A. chroococcum*) kindly provided by Dr. M. Brown at Rothamsted and A_4 (*A. vinelandii*) and A_5 (*A. beijerinckii*), both isolated in this laboratory and studied as plant hormones producers by Azcon and Barea (1975), were used. Inoculum was prepared as before but *Azotobacter* spp. were grown for 14 days as described by Barea and Brown (1974). This mixed culture contained about 10^8 cells/ml.

Soils

The soils were collected from Granada Province, Spain. Soil No. 1 was a "brown calcareous" type (A, B, C), pH = 7.6, containing 30 mg of P, 132 mg of N, and 45 mg of K/kg of soil. Soil No. 8 was a "reddish-brown calcareous" type (A, B, C), pH = 7.6, containing 5 mg of P, 74 mg of N, and 26 mg of K/kg of soil.

Soils also differed in texture: soil No. 1 has 9.70% coarse sand, 22.80% fine sand, 19.25% loam, and 48.22% clay. Soil No. 8 has 8.36% coarse sand, 10.56% fine sand, 11.85% loam, and 70.17% clay. The organic matter content is 1.1% in soil No. 1 and 0.1% in soil No. 8.

Plants

Lavender (*Lavandula spica* L.) was the test plant. Seeds were sown in moistened sand and the seedlings were watered with deionized water and fed with a nutrient solution. Four-week-old seedlings were inoculated by treating their roots with the corresponding inoculum before transplanting to pots containing 250 g of unsterile air-dried soil. Each seedling received about 10^8 phosphobacteria cells or 10^7 *Azotobacter* cells.

Treatments

Half of each soil was amended with 2% (w/w) or farmyard manure. Thus four experimental soils (Nos. 1 and 8, with and without manure) were tested. Twenty replicate pots for each of the four experimental soils were prepared. These 20 pots were divided into four separate lots each receiving a different inoculation treatment.

There were four inoculation treatments: C, control (autoclaved bacterial cultures) (five replications); P, inoculum of phosphate-solubilizing bacteria (five replications); A, inoculum of *Azotobacter* (five replications); and A + P, *Azotobacter* plus "phosphobacteria" (five replications).

Plants were grown for 16 weeks in a glasshouse at 19–25°C, watered from below using a capillarity system.

Measurements

During the experiments rhizosphere soils were sampled at 15-day intervals, as described by Brown *et al.* (1962*b*) and Barea and Brown (1974). About 1.5 g of rhizosphere soil was taken from each of the 80 experimental pots. Then, 10-fold dilution series were prepared for each sample. The first sample was taken 6 weeks after transplanting to avoid possible damage of the tender seedling roots.

The numbers of *Azotobacter* in the suitable dilutions of such samples taken from the five replicate pots of each treatment were counted on a nitrogen-deficient solid medium (Brown *et al.* 1962*a*).

Because of the usual large number of phosphate-solubilizing bacteria in soil, establishment of inoculated ones was only assessed 8 weeks after transplanting and at harvest. Relative idea of this establishment was taken from the comparison of number of phosphate-dissolving bacteria in inoculated and corresponding uninoculated pots. Phosphate-solubilizing bacteria in the above described samples were counted on a solid medium containing 0.02% rock phosphate as described by Barea *et al.* (1970). Solubilization was detected by halo formation around the colonies.

To provide a basis for count, rhizosphere soil was quantified as follows: in the 10^{-1} and 10^{-2} dilutions, soil was recovered, dried at 105°C, and weighed. Bacterial numbers were related to 1 g of dry rhizosphere soil.

At harvest dry weights of roots and shoots were recorded.

Results

Table 1 shows counts of *Azotobacter* recovered from lavender rhizospheres in each experimental soil inoculated with *Azotobacter* alone or in combination with phosphate-solubilizing bacteria. In all cases the numbers of *Azotobacter*

TABLE 1

Numbers of *Azotobacter* in lavender rhizospheres

Soil No.	Organic matter added	Inoculation treatment	Nos. (× 10^3)/g dry rhizosphere soil, weeks after inoculation: 6	8	10	12	16
1	2%	A	1020	292	157	65	9
		A+P	2099**	801***	480**	352***	283***
1	0	A	236	153	18	8	3
		A+P	1160***	250*	44*	27***	7*
8	2%	A	1600	211	143	56	27
		A+P	1500	272	186	120*	55*
8	0	A	901	180	80	77	29
		A+P	1900**	760***	212**	159*	58*

NOTE: A = *Azotobacter*-inoculated pots; P = phosphobacteria-inoculated pots. Significance at 0.1% level (***), 1% level (**), and 5% level (*). A + P vs. A treatments have been compared for each experimental soil.

declined, but at the end of the experiment there were more azotobacters in rhizospheres of plants inoculated with both *Azotobacter* and phosphate-solubilizing bacteria than when *Azotobacter* was inoculated alone. It is clear from Table 1 that in soil No. 1 containing manure, when phosphate-solubilizing bacteria were also inoculated, large *Azotobacter* populations were still present at harvest.

The natural *Azotobacter* population in the two soils and its possible stimulation by phosphate-solubilizing bacteria were assessed during the experiment. Samples were taken from control rhizospheres and phosphate-solubilizing bacteria-inoculated rhizospheres. Table 2 summarizes results obtained 6 weeks after transplanting (first counting). Subsequent counts showed that the natural *Azotobacter* population dropped to a negligible level.

Table 3 gives counts of phosphate-solubilizing bacteria in lavender rhizospheres. In soil No. 8 the introduced inoculum declined by nearly 50% between 8 weeks after inoculation and at harvest.

In soil No. 1, especially when containing manure, populations of phosphate-solubilizing bacteria after 8 weeks and at harvest were similar.

Table 4 summarizes the dry weights of plants grown in the different experimental soils and given different inoculation treatments. Analysis of variance and least significant differences for values given in Table 4 are also shown.

The effect of dual inoculation (*Azotobacter* plus "phosphobacteria") was significant when

TABLE 2

Natural *Azotobacter* population in lavender rhizospheres 6 weeks after inoculation

Soil No.	Organic matter	Nos. (× 10^3)/g dry rhizosphere soil: Control	Phosphobacteria inoculated
1	2%	0.8±0.1	2±0.1
1	0	1 ±0.1	3±0.2
8	2%	5 ±0.3	6±0.4
8	0	4 ±0.3	19±1.2

NOTE: A range of values is given to compare the eight numbers.

organic matter was added, especially in soil No. 1.

Discussion

Azotobacter clearly stimulated the natural population of phosphate-solubilizing bacteria in the root zone comparing C and A treatments (Table 3) and, conversely, phosphobacteria stimulated natural *Azotobacter* population (Table 2).

In manured soil No. 1 (Table 1) *Azotobacter* numbers in the rhizosphere remained high (3–8 × 10^5 *Azotobacter*/g of dry soil) from 8 weeks after inoculation until harvest when phosphobacteria are also inoculated.

Similarly in Table 3, higher numbers of phosphate-solubilizing bacteria were recovered where these bacteria were inoculated with *Azotobacter*, but the numbers were affected by soil fertility. In manured soil No. 1, A + P treatment, "phosphobacteria" establishment was clearly shown.

TABLE 3

Numbers of phosphate-solubilizing bacteria in lavender rhizosphere

Soil No.	Organic matter added	Inoculation treatment	Nos. ($\times 10^3$)/g dry rhizosphere soil, weeks after inoculation: 8	16
1	2%	C	400 ± 35	120 ± 11
		A	750 ± 70	1 600 ± 170
		P	2083 ± 185	2 600 ± 193
		A+P	9400 ± 725	10 000 ± 800
1	0	C	300 ± 31	81 ± 4
		A	673 ± 53	555 ± 33
		P	1530 ± 148	1 650 ± 120
		A+P	4000 ± 275	2 500 ± 190
8	2%	C	375 ± 32	193 ± 12
		A	1730 ± 140	830 ± 51
		P	6000 ± 425	3 900 ± 193
		A+P	7600 ± 412	4 030 ± 180
8	0	C	300 ± 27	114 ± 7
		A	500 ± 31	325 ± 32
		P	1600 ± 120	530 ± 50
		A+P	2600 ± 135	1 000 ± 79

NOTE: C = control, A = *Azotobacter*-inoculated pots, P = phosphobacteria-inoculated pots. A range of values is given.

TABLE 4*a*

Effect of "bacterial fertilizers" on dry weights of lavender plants

Soil No.	Organic matter added	Inoculation treatment	Dry weight (mg) of plants: Shoots	Roots
1	2%	C	230	88
		A	342	209
		P	320	139
		A+P	552	299
1	0	C	220	85
		A	269	174
		P	211	126
		A+P	242	153
8	2%	C	212	102
		A	225	130
		P	229	133
		A+P	330	126
8	0	C	137	89
		A	196	187
		P	127	223
		A+P	199	173

NOTE: C — control; A — *Azotobacter*-inoculated pots; P = phosphobacteria-inoculated pots.

TABLE 4*b*

Analysis of variance for values given in Table 4*a*

Source of variation	Observed F		Required F (Shoots or Roots)		
	Shoots	Roots	0.05 (1)	0.01(1)	0.001 (1)
IT	81.62***	56.71***	2.92	4.51	7.05
OM	179.93***	26.11***	2.92	4.51	7.05
R	1.60	0.30	2.69	—	—
IT × OM	26.76***	14.85***	2.27	3.17	4.58
IT × R	1.81	0.51	2.09	—	—
OM × R	1.12	0.22	2.09		

NOTE: IT, inoculation treatment; OM, organic matter applications; R, replicates, (1) levels of significance, *** Significance at 0.001 level.

TABLE 4*c*

Least significant differences for values given in Table 4*a*

	Shoots	Roots
Between IT		
At 5% level	17	17
At 1% "	24	22
At 0.1% "	31	29
Between additions OM		
At 5% level	17	17
At 1% "	24	22
At 0.1% "	31	29
Interaction IT × OM		
At 5% level	35	33
At 1% "	47	44
At 0.1% "	62	59

The use of both "bacterial fertilizers" in manured soil No. 1 greatly increased plant dry weights. The highest number of introduced bacteria occurred where effects on plant growth were greatest.

However, there have been some apparent anomalies in plant growth. Soil No. 1 did not respond to additions of manure (except when bacteria are added) as noted with soil No. 8. Soil No. 1 had better texture and aeration and it was richer than soil No. 8. The addition of organic matter stimulated the natural microbial population of the soil and microbial processes which in turn affect the availability of soil nutrients. In soil No. 1 competition with the plant for nutrients could account for the lack of response to added organic matter. Conversely, in soil No. 8 organic matter addition improved its texture, and probably compensated for the negative effect of microbial immobilization of nutrients. In spite of that, there were more natural *Azotobacter* cells in soil No. 1 (Table 2), possibly as a result of their protection from lethal factors in the soil by clay minerals, as suggested by Dommergues (1964).

Addition of bacteria to manured soil No. 8 suppressed root growth (as compared to inoculated, unmanured treatments). The inoculated bacteria may produce substances belonging to the auxin gibberellin and cytokinin types, as well as plant-growth inhibitors (Azcon and Barea 1975; Barea, Navarro, and Montoya, to be published). It is known that various growth regulators present at any time may affect growth rate of a plant organ, i.e. root (Thimann 1972). In addition, synthesis of growth substances depends on medium composition; and a rise or fall in concentration affects the response caused by the others. The possibility of a hormonal effect to explain the inhibition of root growth by organic matter addition, in bacteria-inoculated pots, cannot be excluded.

Reviews by Mishustin and Shil'nikova (1971) and Brown (1974) conclude that the positive effect of *Azotobacter* on plant growth cannot be attributed to an enrichment of the medium with bound nitrogen. Similarly, phosphate-solubilizing bacteria have not been shown to increase the overall pool of soluble P in soil. Possibly, at the microhabitat level, some P solubilization, that can improve plant and *Azotobacter* growth, may occur. However, if *Azotobacter* fixes some atmospheric nitrogen, the same mechanism might influence both plant and bacteria.

It is generally accepted that these bacteria by producing plant-growth regulating substances may improve plant growth. Conversely, under some conditions, plant hormones also stimulated microbial development (Lu *et al.* 1958; Saono 1964; Sullia 1968; and others briefly reviewed recently by Barea *et al.* 1974).

The inoculated strains used in this study were selected because of their capacity to produce plant hormones in addition to their N-fixing or P-solubilizing abilities (Barea, Navarro, and Montoya, to be published). From the results obtained it seems that the same cause could account for a positive effect on both higher plants (lavender) and bacteria.

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