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EFFECTS OF ECOLOGICAL FACTORS ON THE ESTABLISHMENT OF *AZOTOBACTER* IN THE RHIZOSPHERE

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Abstract

Several ecological factors which may affect the establishment of an *Azotobacter* population when inoculated into the plant rhizosphere have been tested in experiments carried out in this laboratory.

The present study discusses this problem but deals mainly with: A) The activity of soil bacteriostasis in limiting the growth of *Azotobacter*; and B) The effects of "phosphobacteria" producing plant growth regulating substances.

Azotobacter was sensitive to the bacteriostatic(s) factor(s) of soil from both *Azotobacter*-inoculated and uninoculated pots. Sensitivity of *Azotobacter* increased with time, reached a maximum at the middle of the experiment; then remained almost constant. The activity of this factor seemed to coincide with the drop of *Azotobacter* number in the rhizosphere. The factor was overcome by the addition to the plants of an NPK fertilizer at the middle of the assay.

"Phosphobacteria", which also produce plant hormones, have been reported to stimulate natural *Azotobacter* populations and to influence the establishment of this bacteria when used as inoculum. Consequently, the possibility of such activity being due to the effect of growth substances produced by the phosphobacteria was tested in this study. A decisive influence of the plant hormones present in "phosphobacteria" cultures on *Azotobacter* development was found.

Effects on plant growth of the bacterial "fertilizer" used are also discussed.

Introduction

Inoculation of the plant rhizosphere with *Azotobacter* to improve crop yield has been a topic of much controversy. Thus *A. chroococcum*, first selected because it was believed to fix atmospheric dinitrogen in the root zone, has been widely used. However, this premise has been demonstrated to be incorrect (Brown, 1974).

Although *Azotobacter* has been described as producing increases in plant growth, an effect which is especially marked in fertile soils with a high organic matter content and a neutral pH, its beneficial action is actually associated with the production of biologically active substances (see reviews by Dommergues & Mangenot, 1970; Mishustin & Shilnikova, 1971; Brown, 1974).

In addition to the study of the role of *Azotobacter* in stimulating plant growth another subject attracting much attention is the search for various ecological factors which may affect the establishment of *Azotobacter* when inoculated into the plant rhizosphere.

Ocampo *et al.* (1975), tested three factors that may influence *Azotobacter* establishment in the rhizosphere e.g. soil fertility, manuring, and interactions between *Azotobacter* and phosphate-solubilizing bacteria ("Phosphobacteria"). They found, at harvest, that there were always more *Azotobacter* in the rhizosphere of lavender

(*Lavandula spica* L.) when plants were inoculated with both groups of organisms together. Addition of 2% farmyard manure to the richer soil enhanced this effect. Plant growth also was greatest when seedlings were inoculated with both groups of bacteria.

Phages able to parasitize any of the *Azotobacter* spp. studied (*A. chroococcum*, *A. beijerinckii* and *A. vinelandii*) were not detected during the experiments. However, it was found (Ocampo & Barea, 1975) that *Bdellovibrio* spp. do parasitize the 3 *Azotobacter* spp. Although *Bdellovibrio* seemed to influence the initiation of *Azotobacter* decline in the rhizosphere, it appears as if *Azotobacter* develops a "certain resistance" to being parasitized.

Root extract of lavender inhibited growth and lysed resting cells of *Azotobacter* (Ocampo *et al.*, 1977). This effect increased with the age of the plants.

Amensalism (Stotzky, 1972) was undoubtedly an important factor in the survival and ecology of *Azotobacter* in soil (Ocampo *et al.*, 1978). Microorganisms antagonistic towards growing *Azotobacter* cells, were abundant in the rhizosphere of lavender. These organisms were stimulated when inoculated with *Azotobacter*, but decreased in number at the end of the assay. Microorganisms capable of lysing *Azotobacter* resting cells were also abundant but this was irrespective of the *Azotobacter* inoculation treatment. This activity fluctuated throughout the experiment but was highest at the time of harvest.

Mineral fertilizers stimulated the total microflora and amensalistic microorganisms, but did not affect *Azotobacter* establishment, lytic microorganisms or parasitism by *Bdellovibrio*.

Soil bacteriostasis has been described as an important factor in limiting the growth of soil bacteria (Brown, 1973). Moreover, it was suggested as a cause of the inhibition, under certain conditions, of the germination of *Azotobacter* cysts in soil (Jackson & Brown, 1966).

As Ocampo *et al.* (1975) pointed out, bacteria which produce plant hormones (Barea *et al.*, 1976) stimulated natural and introduced *Azotobacter* populations in the rhizosphere. Plant hormones have also been proved to stimulate growth of other microbes (Lu *et al.*, 1958; Saono, 1964; Sullia, 1968; Barea *et al.*, 1974).

The present paper focuses on: i) The study of soil bacteriostasis on *Azotobacter*, ii) The investigation of the effect of plant hormones on *Azotobacter*.

Material and methods

General experiment

Most of the methods and material used were described by Ocampo *et al.* (1975). Unsterilized field soil No. 1 amended with 2% (w/w) of farmyard manure was used. Lavender (*Lavandula spica* L.) was the test plant. The inocula, seedlings, pots, etc., were prepared as described earlier. Determination of ecological factors affecting *Azotobacter* establishment in the rhizosphere was described in each case (Ocampo *et al.*, 1977, 1978).

Determination of soil bacteriostatic activity

For assessing soil bacteriostasis to *Azotobacter* a technique based on that described by Brown (1973) was used. Soils from *Azotobacter*-inoculated rhizosphere and uninoculated control rhizosphere were compared with controls without soil. Rhizosphere soil from each sample was placed in Petri dishes and moistened to 70% of field capacity. Disks of Whatman No. 1 filter paper were either placed on the soil, or in sterile dishes

as controls. Agar-disks 7 mm in diameter (9 replicates per sample), cut from 1.5% sterile distilled water agar, were placed on the filter paper. Dishes were kept at 26°C for 15 h and were then inoculated with 0.01 ml of suspensions of each of the three *Azotobacter* spp. (*A. chroococcum*, A₆; *A. beijerinckii*, A₂ and *A. vinelandii*, A₃). Three replicates per *Azotobacter* spp. were prepared. After 48 h incubation at 26°C, the disks were removed, stained with 10% diluted carbon fuchsin and examined under the microscope. Bacteriostasis was assessed by comparing the number of micro-colonies which grew on disks incubated over soil with those on the controls.

Effect of plant hormones produced by phosphobacteria on *Azotobacter* development

This was studied both *in vitro* and in a pot experiment:

i) *Effect in vitro*. - Six conical flasks containing N-deficient liquid medium, prepared as described by Barea & Brown (1974) were each inoculated with 1 ml of a suspension of *Azotobacter* (A₆) cysts in sterile distilled water. Two of these flasks were also inoculated with 1 ml of a phosphobacteria culture (PB treatment) prepared as described by Ocampo *et al.* (1975). This culture contained about 0.1 µg of each of the plant hormones auxins, gibberellins and cytokinins (Barea *et al.*, 1976). One ml of a mixture of commercial hormones at the mentioned concentrations was added to another two flasks (PH treatment). Numbers of *Azotobacter* in the three treatments were counted after 1, 3 and 5 days of incubation at 26°C on a rotary shaker.

ii) *Effect in plant rhizosphere*. - Lavender seedlings were inoculated by treating their roots with the inocula mentioned above (A₆, A₆ + PB and A₆ + PH), and were cultivated as described by Ocampo *et al.* (1975). During the experiment, rhizosphere soil was sampled at 15-day intervals and *Azotobacter* counted, as described by Barea & Brown (1974).

Results and discussion

Table 1 shows that the three *Azotobacter* spp. were sensitive to bacteriostatic factors in lavender rhizosphere soil from both *Azotobacter* inoculated and uninoculated pots. As Fig. 1 more clearly shows, sensitivity of *Azotobacter* increased with time, and reached a maximum at the middle of the experiment; then remained almost constant. The living roots behaved as if supplying substances to help to overcome the bacteriostasis towards *Azotobacter*. Bacteriostasis was also overcome by the addition to the soil of a NPK fertilizer at the middle of the assay. This was also found by Jackson (1960) and Brown (1973). The exact nature of the bacteriostatic factors is still unknown, but Brown's finding suggests that it could be of biological origin.

Tables 2 & 3 show that plant hormones play a certain role in *Azotobacter* development both *in vitro* and in the rhizosphere. Phosphate-solubilizing bacteria, however, may act by another mechanism in addition to that based on the supply of hormones.

Figure 1 also shows that the course of development of the activity towards *Azotobacter* of soil bacteriostasis coincides with the numerical decline of *Azotobacter* in the rhizosphere. The period at which that antagonistic factor is expressed is similar to that previously found for other antagonistic agents (Ocampo *et al.*, 1975, 1977, 1978), and it is difficult to elucidate which of these factors plays the major role in antagonizing *Azotobacter* in the rhizosphere. The lack of conclusive evidence for most of the mechanisms, *in situ*, is a general trend (Stotzky, 1972).

Regardless of the mechanisms involved, the *Azotobacter* population introduced into the lavender rhizosphere was proved to suffer from the activity of several ecological factors which govern the biological equilibria in the root region. However, a number of *Azotobacter* become established. Initially (between 1 and 6 weeks after inoculation) *Azotobacter* was stimulated but after 6 to 12 weeks cell numbers dropped; finally, cell

Table 1. Effect of soil bacteriostatic factors on *Azotobacter* in control and *Azotobacter*-inoculated rhizosphere.

Weeks after inoculation	Inoculation treatment	% of colonies*		
		A ₄ ***	A ₅	A ₆
2	Control (C)	59	62	56
	Azotobacter (A)	44	43	45
4	C	57	60	51
	A	40	39	44
7	C	47	50	52
	A	39	35	43
10	C	51	45	45
	A	36	34	41
13	C	52(63)**	50(61)	48(61)
	A	39(67)	33(63)	42(65)
16	C	53(82)	52(80)	54(72)
	A	42(96)	37(94)	32(89)

* Percent colonies in relation to controls with no soil added.

** The parentheses contain % of the numbers of colonies in NPK treated rhizosphere.

*** A₄ = *A. beijerinckii*; A₅ = *A. vinelandii* and A₆ = *A. chroococcum*.

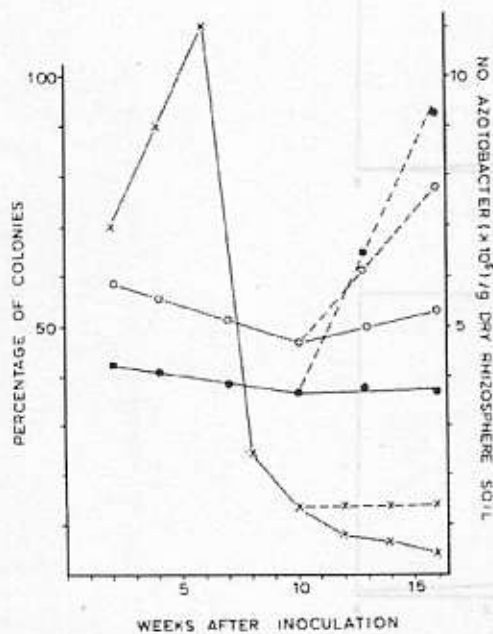


Figure 1. Course of development of *Azotobacter* (x—x) no. · g⁻¹ dry rhizosphere soil, and bacteriostatic activity towards *Azotobacter* (% of colonies) in control (o—o) and inoculated (●—●) rhizosphere, affected by NPK fertilizer (—).

Table 2. Effect of phosphobacteria (PB) and plant hormones (PH) on numbers of *Azotobacter* (A) in culture.

Inoculation treatment	No. ($\times 10^7$) ml culture ⁻¹ (Age of culture, days)		
	1	3	6
A	16.5	3.5	3.3
A + PB	31.5	15.5	15.4
A + PH	33.5	17.5	17.2
L.S.D. (5%)	5.5	2.5	2.5

L.S.D. (5%) = Least Significant Difference at 5%.

Table 3. Effect of phosphobacteria (PB) and plant hormones (PH) on numbers of *Azotobacter* (A) inoculated in lavender rhizosphere.

Inoculation treatment	No. ($\times 10^5$) g dry rhizosphere soil ⁻¹ (weeks after inoculation)							
	2	4	6	8	10	12	14	16
A	44	90	140	24	13.2	4.9	3.6	3
A + PB	125	130	200	65	50	23	24	19
A + PH	111	120	189	47	33	10	11	7
L.S.D. (5%)	16	17	30	9.8	8.2	7.3	6.4	2.9

L.S.D. (5%) = Least Significant Difference at 5%.

numbers became equilibrated. In the present experiments each seedling received about 10^7 *Azotobacter* cells which were stimulated to 10^7 g dry rhizosphere soil⁻¹, and 10^5 – 10^6 cells g⁻¹ remained at harvest.

Table 4 summarizes the dry weights of plants grown in the different experimental conditions and given different inoculation treatments.

Table 4. Effect of bacterial "fertilizers" on dry weights of lavender plants as effected by NPK fertilizer.

Inoculation* treatment	NPK treatment** (mg plant ⁻¹)			
	1	2	3	4
C	600	885	881	1022
A	720	1025	995	1187
PB	660	913	1045	1153
A + PB	780	1085	1090	1176
L.S.D. (5%)	55	60	50	70

* C = Uninoculated control; A = *Azotobacter*-inoculated pots; PB = phosphobacteria-inoculated pots.
 ** 1 = no NPK added; 2 = NPK added at inoculation time; 3 = NPK added at the middle of assay; 4 = 2 + 3 treatment.

L.S.D. (5%) = Least Significant Difference at 5%.

In NPK treatments 1, 2 and 3, but not in 4; there were more *Azotobacter* at harvest when plants were inoculated with *Azotobacter* together with phosphobacteria than when inoculated singly. Although this is apparently reflected in Table 4, in which differences between plant dry weights in A + P vs. A treatment are significant in 1, 2 and 3 treatments, but not in 4; this may be a direct effect of phosphobacteria on plant growth.

The *Azotobacter* tested (Brown & Burlingham, 1968; Azcón & Barea, 1975) and the phosphobacteria (Barea *et al.*, 1976) can produce *in vitro* plant growth substances which could play a role in improving plant growth, as has been suggested (Brown, 1974).

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