Interactions between *Azobacter* and "phosphobacteria" and their establishment in the rhizosphere as affected by soil fertility

J. A. Ocana, J. M. Barba, and E. Montoya

Microbiology Department, Experimental Station of Lusit, Granada, Spain

Accepted April 11, 1975


The effect on plant growth of "bacterial fertilizers" prepared from *Azobacter* 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, mining, and interactions between *Azobacter* and "phosphobacteria," were examined in pot experiments, designed for statistical analysis, in two neutral-alkaline soils, using lavender-plagio (Lavandula angustifolia L.). After the experiments the numbers of *Azobacter* and "phosphobacteria" were counted. The growth of roots and shoots were recorded after 16 weeks or growth.

As the end of the experiment, there were new *Azobacter* 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 soil reduced the effect. The growth was greater when the inoculations were inoculated with both *Azobacter* and the "phosphobacteria."


Les effets sur la croissance des plantes de bactéries fertilisantes préparées à partir d’*Azobacter* spp. et de bactéries solubilisant le phosphore ("phosphobacteria") ont été le sujet de plusieurs controverses. Les cas où il n’y a pas de stimulation de la croissance des plantes peuvent souvent être expliqués par le manque d’établissement des泓atéries bactériennes dans le rhizosphere.

Nous avons étudié trois facteurs qui peuvent influencer l’établissement des inoculants, i.e., la fertilité du sol, la fertilité et les interactions entre *Azobacter* et "phosphobacteria," dans des expériences en pot désignées pour des analyses statistiques dans deux sols neutre-alkalins, un cultivé de lavande-plagio (Lavandula angustifolia L.). Au cours des expériences les nombres d’*Azobacter* et de phosphobacteria furent comptés. Les plantes avec des racines et des tiges furent déterminées après 16 semaines de croissance.

À la fin des expériences, il y avait toujours plus d’*Azobacter* et de "phosphobacteria" dans les rhizosphères que 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 accentua cet effet.

La croissance des plantes fut plus grande lorsque les sémens furent inoculés avec les deux cultures *Azobacter* 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 *Azobacter*, *Rhizobium*, and phosphate-solubilizing bacteria ("phosphobacteria") according to Ramos-Cormazana (1970) have been used extensively (see reviews by Domergue and Mangerot 1970; Mcsustin and Shillington 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. *Azobacter* or "phos-
phenobacteria," 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 "phenobacteria," 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 Arthrobacter sp., and a Bacillus sp. were selected from 100 million dilution solutions of sodium phosphate and rock phosphate, in pure culture (these bacteria produce plant-growth hormones, according to Barer, Navarro, and Monafo, to be published). Inoculum was prepared by growing each bacterium in the medium of Brown (1973) at shake-culture for 10 days at 23°C. At the time of inoculation the three species were combined. This mixed culture contained about 10³ cfu/ml.

Three Azotobacter strains, A. chroococcum, kindly provided by Dr. M. Brown at Rothamstead and A. chroococcum and A. beijerinckii, both isolated in the laboratory and studied as plant hormones producers by Azcon and Barra (1975), were used. Fosculum was prepared as before but Azotobacter spp. were grown for 24 days as described by Barca and Brown (1974). This mixed culture contained about 10³ cfu/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, 146 mg of N, and 45 mg of K/kg of soil. Soil No. 2 was a "red-brown calcareous" type (A, B, C), pH = 7.6, containing 3 mg of P, 24 mg of N, and 35 mg of K/kg of soil.

Soils also differed in texture: soil No. 1 has 9.30% coarse sand, 23.00% fine sand, 12.00% loam, and 17.25% clay. Soil No. 2 has 8.26% coarse sand, 50.20% fine sand, 13.25% loam, and 18.75% clay. The organic matter content is 1.7% in soil No. 1 and 0.6% in soil No. 2.

Plants

Lavender (Lavandula spice) was the test plant. Seeds were sown in sterilized sand and the seedlings were watered with distilled water and tea with a nutrient solution. Four-week-old seedlings were inoculated by treating their roots with the corresponding inoculum before transplanting to pots containing 200 g of sterile air-dried soil. Such soil received about 10³ phosphate-solubilizing cells or 10³ Azotobacter cells.

Treatments

Half of each soil was amended with 2% (w/w) of farmyard manure. Thus four treatments (No. 1, 2, 3, and 4, 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 replicate 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 "phenobacteria" (five replications).

Plants were grown for 16 weeks in a greenhouse at 19-25°C, watered from below using a capillary system.

Measurements

During the experiment, rhizosphere soils were sampled at 13-day intervals, as described by Brown et al. (1962) and Barca 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 8 weeks after transplanting to avoid possible damage from 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).

Because of the usual large number of phosphate-solubilizing bacteria in soil, establishment of inoculated ones was only measured 3 weeks after transplanting and at harvest. Relative idea of this establishment was taken from the comparison of number of phosphate-solubilizing 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 Barca et al. (1970). Solubilization was detected by halo formation around the colonies.

To provide data for analysis, 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.

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

<table>
<thead>
<tr>
<th>Soil No.</th>
<th>Organic matter added</th>
<th>Inoculation treatment</th>
<th>Numbers of Azotobacter per 100 g dry rhizosphere soil, weeks after inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>1</td>
<td>2%</td>
<td>A</td>
<td>1020</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A + P</td>
<td>2000***</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>A</td>
<td>250</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A + P</td>
<td>1100***</td>
</tr>
<tr>
<td>5</td>
<td>2%</td>
<td>A</td>
<td>1100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A + P</td>
<td>1500</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>A</td>
<td>900</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A + P</td>
<td>1900***</td>
</tr>
</tbody>
</table>

Notes: A = Azotobacter inoculated; P = Phosphate-solubilizing soil. *Significant at 0.1%, ** at 0.01%, *** at 0.001%. A + P means 2 treatments have been adopted for each experimental soil.

TABLE 2

<table>
<thead>
<tr>
<th>Soil No.</th>
<th>Organic matter</th>
<th>Control</th>
<th>Phosphate-solubilizing bacteria inoculated</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0%</td>
<td>0.0 ± 0.1</td>
<td>2.0 ± 0.1</td>
</tr>
<tr>
<td>1</td>
<td>2%</td>
<td>1.0 ± 0.1</td>
<td>3.0 ± 0.3</td>
</tr>
<tr>
<td>2</td>
<td>2%</td>
<td>0.5 ± 0.2</td>
<td>6.0 ± 0.4</td>
</tr>
<tr>
<td>5</td>
<td>0%</td>
<td>0.4 ± 0.3</td>
<td>10 ± 1.2</td>
</tr>
</tbody>
</table>

Notes: A range of values is shown to emphasize the effect numbers. Organics matter was added, especially in soil No. 1.

Discussion

Azotobacter clearly stimulated the natural population of phosphate-solubilizing bacteria in the root zone of plant A and treatment (Table 3) and, conversely, phosphate-bacteria stimulated natural Azotobacter population (Table 2).

In measured soil No. 1 (Table 1) Azotobacter numbers in the rhizosphere remained high (3-8 x 10^6 Azotobacter/g of dry soil) from 8 weeks after inoculation until harvest when phosphate-bacteria 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 measured soil No. 1, A + P treatment, "phosphate-bacteria" establishment was clearly shown.
### TABLE 3

Numbers of phosphate-solubilizing bacteria in lavender rhizosphere

<table>
<thead>
<tr>
<th>Soil No.</th>
<th>Organic matter added</th>
<th>Inoculation treatment</th>
<th>Nos. (× 10^9)/g dry rhizosphere soil, weeks after inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>8</td>
</tr>
<tr>
<td>1</td>
<td>2%</td>
<td>C</td>
<td>400 ± 35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A</td>
<td>790 ± 70</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P</td>
<td>2083 ± 185</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A + P</td>
<td>9400 ± 725</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>C</td>
<td>300 ± 31</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A</td>
<td>673 ± 53</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P</td>
<td>1530 ± 148</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A + P</td>
<td>4000 ± 275</td>
</tr>
<tr>
<td>8</td>
<td>2%</td>
<td>C</td>
<td>375 ± 32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A</td>
<td>1730 ± 140</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P</td>
<td>6000 ± 425</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A + P</td>
<td>7600 ± 412</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>C</td>
<td>300 ± 27</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A</td>
<td>500 ± 31</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P</td>
<td>1600 ± 120</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A + P</td>
<td>2600 ± 135</td>
</tr>
</tbody>
</table>

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

### TABLE 4a

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

<table>
<thead>
<tr>
<th>Soil No.</th>
<th>Organic matter added</th>
<th>Inoculation treatment</th>
<th>Dry weight (mg) of plants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Shoots</td>
</tr>
<tr>
<td>1</td>
<td>2%</td>
<td>C</td>
<td>230</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A</td>
<td>342</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P</td>
<td>320</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A + P</td>
<td>552</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>C</td>
<td>220</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A</td>
<td>269</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P</td>
<td>211</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A + P</td>
<td>242</td>
</tr>
<tr>
<td>8</td>
<td>2%</td>
<td>C</td>
<td>212</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A</td>
<td>225</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P</td>
<td>229</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A + P</td>
<td>330</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>C</td>
<td>137</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A</td>
<td>196</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P</td>
<td>127</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A + P</td>
<td>199</td>
</tr>
</tbody>
</table>

Note: C = control; A = Azotobacter-inoculated pots; P = phosphobacteria-inoculated pots.
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 were 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 Demmenges (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 azin, glucorhin, 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 (Thimmann 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 Kishustin and Shilnikova (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 microbial 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

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Shoots</th>
<th>Roots</th>
<th>0.05 (1)</th>
<th>0.01 (1)</th>
<th>0.001 (1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IT</td>
<td>51.52***</td>
<td>56.11***</td>
<td>2.92</td>
<td>4.51</td>
<td>7.05</td>
</tr>
<tr>
<td>OM</td>
<td>199.39***</td>
<td>26.11***</td>
<td>2.92</td>
<td>4.51</td>
<td>7.05</td>
</tr>
<tr>
<td>R</td>
<td>1.60</td>
<td>0.30</td>
<td>2.69</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>IT × OM</td>
<td>16.76***</td>
<td>14.38***</td>
<td>2.77</td>
<td>4.17</td>
<td>5.48</td>
</tr>
<tr>
<td>IT × R</td>
<td>1.81</td>
<td>0.51</td>
<td>2.59</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>OM × R</td>
<td>1.15</td>
<td>0.32</td>
<td>2.29</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

*Critical values for significance at 0.05 level.

TABLE 4b

Least significant differences for values given in Table 4b.
microbial development (Lu et al. 1958; Sacco 1964; Sullivan 1968; and others briefly reviewed recently by Bara et al. 1974).

The inoculated strains used in this study were selected because of their capacity to produce plant hormones in addition to their nitrogen or phosphobuilding abilities (Bara, Navarro, and Moroney, to be published). From the results obtained it seems that the same cause could account for a positive effect on both higher plants (flaxseed) and bacteria.

Acknowledgments

The authors thank Dr. M. Gómez-Osaba, Experimental Station of Zaidin, Granada, Spain, for analysis of variance of the results given in Table 4.

The authors' gratitude is extended to Dr. D. S. Hayman, Rothamsted Experimental Station, Harpenden, England, for the grammatical correction of this manuscript.


