Possible Involvement of Phage-Like Structures in Antagonism of Cowpea Rhizobia by *Rhizobium trifolii*

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A reduction in the viability of cowpea rhizobia was observed when *Rhizobium trifolii* IARI and cowpea *Rhizobium* strain 3824 were inoculated together in soil. The reduction in number of cowpea rhizobia in soil was found to be associated with the reduction in number of nodules per plant and retardation in plant growth. An antimicrobial substance was isolated from *R. trifolii* which, on electron microscopic investigation, demonstrated the presence of several phage-like structures.

The members of the genus *Rhizobium* possess the ability to survive and multiply in soil and to compete in nodule formation (4–6, 8). Although the competitive abilities of *Rhizobium* strains have received much attention in various laboratories (1, 17), the antagonistic effect of various *Rhizobium* species during nodulation has been poorly exploited. Antagonism may affect symbiosis by either reducing survival and multiplication of *Rhizobium* spp. in soil and rhizosphere or inhibiting the infection process for nodulation.

Earlier work from this laboratory demonstrated the production of an antimicrobial substance (AMS) and a bacteriocin-like substance by *Rhizobium trifolii* strains IARI and Rel-1, respectively (9; M. V. Joseph, Ph.D. thesis, Sardar Patel University, Vallabhb-Vidyanyagar, India, 1983). The AMS showed a wide host range activity, and we speculated upon the ecological significance of this phenomenon for the survival of the producer strain. The present report, an extension of our earlier work (9), concentrates on the effect of *R. trifolii* on survival of cowpea rhizobia and growth of cowpea plants.

*R. trifolii* strains IARI and RCR-5 and cowpea *Rhizobium* strain 3824 and their maintenance were the same as described earlier (9). Nodulation experiments were performed as described by Wacek and Brill (18), with the following modifications. Cowpea seeds were surface sterilized and allowed to germinate for 2 days on moistened filter paper kept in sterile petri plates held in the dark at 30°C. Germinated seed was planted in each Leonard jar (12) containing autoclaved soil and irrigated with nitrogen-free nutrient solution. Plants were grown in an environmental growth chamber for 16 h in the light and 8 h in the dark at 24°C. Six weeks later, nodulation was observed.

A mutant strain of cowpea *Rhizobium* strain 3824 resistant to streptomycin sulfate (300 µg/ml) and sodium azide (200 µg/ml) was isolated with N-methyl-N'-nitro-N-nitrosoguanidine as a mutagenic agent (10). *R. trifolii* (an AMS producer) was inoculated together with this mutant in a desired proportion to investigate its ecological effect on nodulation. The viability of cowpea rhizobia in the presence of the AMS producer *R. trifolii* was checked on yeast extract-mannitol

![FIG. 1. Growth of cowpea plant in the absence (A) and presence (B) of 8 x 10⁶ cells of R. trifolii. Experimental conditions were as described in Table 1. Bars, 3 cm.](http://aem.asm.org/download/fig1.png)

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TABLE 1. Effect of R. trifolii on nodulation and dry weight of cowpea plants

<table>
<thead>
<tr>
<th>Culture supplement</th>
<th>No. of nodules per plant (mean ± SD)</th>
<th>Dry wt (g) (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cowpea rhizobia (control)</td>
<td>67 ± 0.81</td>
<td>2.7 ± 0.050</td>
</tr>
<tr>
<td>Cowpea rhizobia + R. trifolii IARI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:1</td>
<td>32 ± 1.25</td>
<td>1.7 ± 0.021</td>
</tr>
<tr>
<td>1:2</td>
<td>19 ± 0.81</td>
<td>1.4 ± 0.024</td>
</tr>
<tr>
<td>1:3</td>
<td>8 ± 0.70</td>
<td>1.2 ± 0.031</td>
</tr>
<tr>
<td>1:4</td>
<td>4 ± 0.5</td>
<td>0.8 ± 0.020</td>
</tr>
<tr>
<td>1:5</td>
<td>0 ± 0.5</td>
<td>0.7 ± 0.031</td>
</tr>
<tr>
<td>Cowpea rhizobia + R. trifolii RCR-5</td>
<td>65 ± 1.41</td>
<td>2.8 ± 0.036</td>
</tr>
</tbody>
</table>

* The nodulation experiment was performed as described in the text, with cowpea Rhizobium strain 3824 and a specific host plant. Two milliliters of cowpea rhizobia (10⁷ cells per ml) was inoculated into the rhizosphere after the germinated seeds were planted in a Leonard jar (12). The experimental jars received an additional inoculum of R. trifolii as indicated. During mixed inoculation, R. trifolii RCR-5 (a non-AMS producer) served as an additional control. The total volume inoculated was kept constant. The values shown were obtained after 6 weeks under culture conditions.

FIG. 2. Effect of R. trifolii on the viability of cowpea rhizobia in soil. A total of 25 g of sterile soil was inoculated with 10 ml of nitrogen-free nutrient solution containing 5 × 10⁸ cowpea rhizobia cells and R. trifolii in proportions of 1:1 (a), 1:2 (b), 1:3 (c), 1:4 (d), and 1:5 (e). The inoculum size was kept constant. The soil was mixed thoroughly after inoculation and incubated at 24°C. At desired time intervals, samples were withdrawn and viable counts of cowpea rhizobia on plates containing streptomycin (300 µg/ml) and sodium azide (200 µg/ml) were made. Inocula without (●) and with (○) R. trifolii RCR-5 (a non-AMS producer) were considered controls.

FIG. 3. Electron micrographs of AMS preparation from R. trifolii IARI. AMS production was followed as described previously (9). For electron microscopic observation, the inhibition zone was cut from L-arabinose agar and homogenized in 0.1 M phosphate buffer (pH 7) containing 1 M NaCl. After centrifugation at 5,000 × g for 15 min, the supernatant was passed through a membrane filter (pore size, 0.45 µm; Millipore Corp.) and stored at 4°C over a layer of chloroform. The filtrate was negatively stained with 20% uranyl acetate on Formvar- and carbon-coated grids. Grids were air dried in a desiccator for 1 h and observed under a Philips 400 electron microscope.

The results (Table 1) show a significant reduction in nodule formation on the cowpea plant when R. trifolii was inoculated together with cowpea rhizobia in soil. Interestingly, the reduction in the number of nodules formed was found to be proportional to the increase in the size of the R. trifolii inoculum. A significant reduction in the growth of the cowpea plant was observed when R. trifolii was inoculated together with cowpea rhizobia as compared with the growth of the plant which received only cowpea rhizobia (Fig. 1).
Similarly, the dry matter of the plant decreased considerably when the soil was supplemented with *R. trifolii* during the growth of the cowpea plants, and this reduction was directly proportional to the amount of *R. trifolii* cells used to supplement the soil (Table 1).

To quantitate the antagonistic effect of *R. trifolii* on cowpea rhizobia, we examined the viable population of cowpea rhizobia in soil after mixed inoculation. A mutant strain of cowpea *Rhizobium* strain 3824 that is resistant to streptomycin sulfate and sodium azide was used for this experiment (Fig. 2). The viable counts of cowpea rhizobia decreased in soil; this reduction was proportional to the density of *R. trifolii* mixed into the soil. The jars which received only cowpea rhizobia and *R. trifolii* RCR-5 (a non-AMS producer) plus cowpea rhizobia demonstrated an increase in the number of cowpea rhizobia with time. This rules out the possibility of any other factor being responsible for the reduction in number of cowpea rhizobia during mixed inoculation with *R. trifolii*. If one considers the increase in the population of cowpea rhizobia under control conditions, the antagonistic effect of *R. trifolii* on cowpea rhizobia is much more profound than is depicted in Fig. 2. The mutant used in this experiment was not deficient in nodule formation and supported the growth of cowpea plants as well.

The electron microscopic observation of the AMS preparation obtained from *R. trifolii* IARI showed the presence of phage-like structures (Fig. 3). Several investigators have shown the production by *Rhizobium* sp. of bacteriocins that resemble phage-like structures (11, 13–15). The production of phages by *R. trifolii* strains is also documented in the literature (2, 3, 16). Evans et al. (7) noted the inhibitory effect of *R. trifolii* phages on nodulation.

Thus, the data presented in this paper lead us to suggest that *R. trifolii* IARI had an antagonistic effect on cowpea rhizobia which in turn may have been responsible for the reduction in formation of nodules during mixed inoculation and eventually retarded the growth of the plant. The antagonistic effect of *R. trifolii* IARI on cowpea rhizobia may be attributed to the AMS produced by *R. trifolii*, which was found to contain phage-like structures.

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**LITERATURE CITED**


