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Erwinia herbicola Isolates from Alfalfa Plants May Play a Role in Nodulation of Alfalfa by *Rhizobium meliloti*

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Erwinia herbicola **was isolated from roots of plants derived from surface-sterilized seeds of all alfalfa varieties that were tested. Some of these *E. herbicola* strains affected nodulation by certain strains of *Rhizobium meliloti*. In previously published work we presented the isolation of slow-and fast-nodulating variants from a single culture of *R. meliloti* 102F51. In the absence of *E. herbicola*, the slow-nodulating variant induced the formation of nodules on alfalfa as rapidly as the faster-nodulating strain. The rates of nodulation by the faster-nodulating variant were the same in the presence and absence of *E. herbicola*. All of the previously reported slower-nodulating strains derived from *R. meliloti* 102F51 nodulated more rapidly on sterilized plants than in the presence of certain *E. herbicola* isolates.**

After years of applying rhizobia inoculants to the soil, farmers are faced with the problem that indigenous *Rhizobium* strains have become highly competitive but may not fix as much nitrogen as commercial inoculants. For this reason highly competitive, efficient nitrogen-fixing inoculants are needed. To produce these inoculants, factors that influence nodulation and competition between rhizobia should be understood.

One factor that may affect nodulation by *Rhizobium* strains is the presence of other organisms in the rhizosphere. B. Bohlool has shown that the competitiveness of some *R. japonicum* strains is different in sterile and nonsterile soil (unpublished data). There are also reports that demonstrate antagonism of other soil organisms to rhizobia (4, 8–10), but isolated bacteria have not been shown to inhibit nodulation. Here we demonstrate that the presence of a common, epiphytic bacterium, *Erwinia herbicola*, which is present in almost all alfalfa seeds, affects the nodulation of alfalfa by some *R. meliloti* strains.

MATERIALS AND METHODS

Seed sterilization. Alfalfa seeds (*Medicago sativa* L.) cultivar Vernal were obtained from Olds Seed Co., Madison, Wis., and the other cultivars were obtained from Stanley Duke, University of Wisconsin. Alfalfa seeds were soaked in concentrated sulfuric acid for 15 min, washed with 3 liters of sterile distilled water, and placed in sterile distilled water to germinate. They were shaken overnight, and then the water was replaced with either sterile distilled water or 50 μg of CGA78039 per ml (1; the kind gift of Arthur Kelman) in 0.005% ammonium hydroxide. After 6 h more of shaking, the germinated seeds were washed with sterile distilled water and shaken overnight in sterile distilled water. The seedlings were placed on sterile filter paper in large glass petri plates and after 24 h were placed on microscope slides in distilled water, covered with a cover slip, and observed with phase-contrast optics with a Zeiss photomicroscope. Seedlings were placed on yeast extract-mannitol agar plates (6) to check sterility.

Nodulation. After the second overnight incubation in distilled water, the seedlings were planted in individual sterile vermiculite-filled vials as described previously (6). Each data point (see Fig. 2) represents the mean number of nodules on 40 plants. Since the plants were removed from the vermiculite vials, each point represents a different group of 40 plants. All cultures were grown for 3 days in yeast extract-mannitol medium as previously described (6). Nodule bacteria were recovered and identified as described previously (3).

RESULTS

Erwinia herbicola was found in at least some of the seeds of all of the alfalfa varieties that were tested. The seeds were surface sterilized with concentrated sulfuric acid and germinated on yeast extract-mannitol agar plates. After the seed coats had broken, yellow mucoid growth was found around the emerging seedlings. When this material was streaked on agar plates, it was found to contain only one type of organism, which was identified as the bacterium *E. herbicola* according to *Bergey's Manual of Determinative Bacteriology* (5). Table 1 shows the proportion of seeds of eight varieties of alfalfa that contained *E. herbicola*. All of the seeds, except for some of variety NC8376, contained *E. herbicola*.

Antibiotics such as penicillin, ampicillin, tetracycline, chloramphenicol, erythromycin, bacitracin, sulfadiazine, and nalidixic acid were used to try to eliminate the bacteria from alfalfa seedlings; however, these compounds caused morphological aberrations or greatly decreased the growth rates of the plants. The bacteriocide CGA78039 (1) eliminated the *E. herbicola* from 1-day-old plants that were exposed to a solution containing 50 μg/ml for 6 h. The plants were completely free of bacteria, as determined by plating them on yeast extract-mannitol medium and by microscopic examination. Figure 1 shows phase-contrast photomicrographs of the root hairs of plants which had been germinated in sterile distilled water (1A) or treated with CGA78039 (1B).

We have previously described *R. meliloti* strains that were derived from strain 102F51 and which are agglutinated at

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TABLE 1. Presence of *E. herbicola* in alfalfa seeds

Alfalfa variety	No. contaminated/ no. tested
M. sativa Vernal	20/20
M. sativa Sonora	10/10
M. sativa Ranger	20/20
M. sativa Hairy Peruvian	20/20
M. sativa NC8376	10/20
M. sativa Saranac	20/20
M. sativa Iroquois	20/20
M. falcata	12/12

different concentrations of the alfalfa agglutinin (3). The more highly agglutinable (HA) strains nodulate more slowly than the less agglutinable (LA) strains. We determined the rate of nodulation of two of these strains on sterilized plants and on sterilized plants which had been inoculated with *E. herbicola* isolated from Vernal alfalfa plants. Figure 2B shows that nodulation by the LA strain WL100 was unaffected by the presence of *E. herbicola*, whereas nodulation by the HA strain WL200 (Fig. 2A) was slowed down by *E. herbicola*. In the absence of *E. herbicola*, the HA strain nodulated as rapidly as the LA strain.

Nodulation by a variety of HA and LA strains was studied in the presence and absence of the *E. herbicola* isolate from Vernal alfalfa (Table 2). These strains, selected by phage resistance (3), behaved similarly to strains WL100 and WL200. Fifty sterilized plants were inoculated with each HA or LA strain in the absence or presence of *E. herbicola*, and plants were examined six days after inoculation. All five HA strains induced significantly more nodules (at the 95% confidence level by Student's *t* test) in the absence than in the presence of *E. herbicola*. There was no significant difference between the number of nodules produced by the LA strains in the presence or absence of the *E. herbicola*, except for strain WL101, which induced more nodules in the presence of *E. herbicola*. We have no explanation for the behavior of strain WL101. The number of nodules induced in both the presence and absence of *E. herbicola* by each strain was determined in the same experiment, so that these comparisons are valid even if experiments with different strains cannot be compared with each other.

E. herbicola isolates from other varieties of alfalfa were also examined for their ability to inhibit nodulation by the HA strain WL200 on Vernal alfalfa. Table 3 shows that in the presence of *E. herbicola* isolates from the alfalfa varieties Vernal and Sonora and another species of alfalfa, *Medicago falcata*, strain WL200 induced significantly fewer nodules than did strain WL100, whereas there was no significant difference between the number of nodules induced by these strains on sterilized plants and the number induced on plants to which four other isolates of *E. herbicola* had been added.

The LA strains were previously shown to be more competitive than the HA strains in mixed inoculum experiments (3). Since the HA strains nodulated as rapidly as the LA strains on sterilized plants, it seemed likely that the LA strains would also lose their competitive advantage on sterile plants. The LA strain, WL100, comprised 78 ± 8.5% of the nodule isolates of 20 plants in a mixed-inoculum experiment performed as described previously (3) on sterilized plants. When *E. herbicola* was added to the plants (10^8 cells per plant) before inoculation with *R. meliloti*, the LA cells comprised 97 ± 1.6% of the nodule isolates. Although the LA cells did not completely lose their competitive advantage on sterilized plants, the presence of *E. herbicola* decreases the competitiveness of the HA strain.

Addition of *E. herbicola* to sterilized plants causes a significant increase in the proportion of LA isolates in the nodules. Thus, the *E. herbicola* influences competitiveness of the HA strains in addition to or because it decreases the rate of nodulation by the HA cells. It is not clear why the LA strain WL100 has some competitive advantage even on sterilized plants, since we assumed that the competitiveness was due to the early nodulating phenotype.

DISCUSSION

It is interesting that *E. herbicola* is so widespread in alfalfa seeds. *E. herbicola* has been found previously in a wide

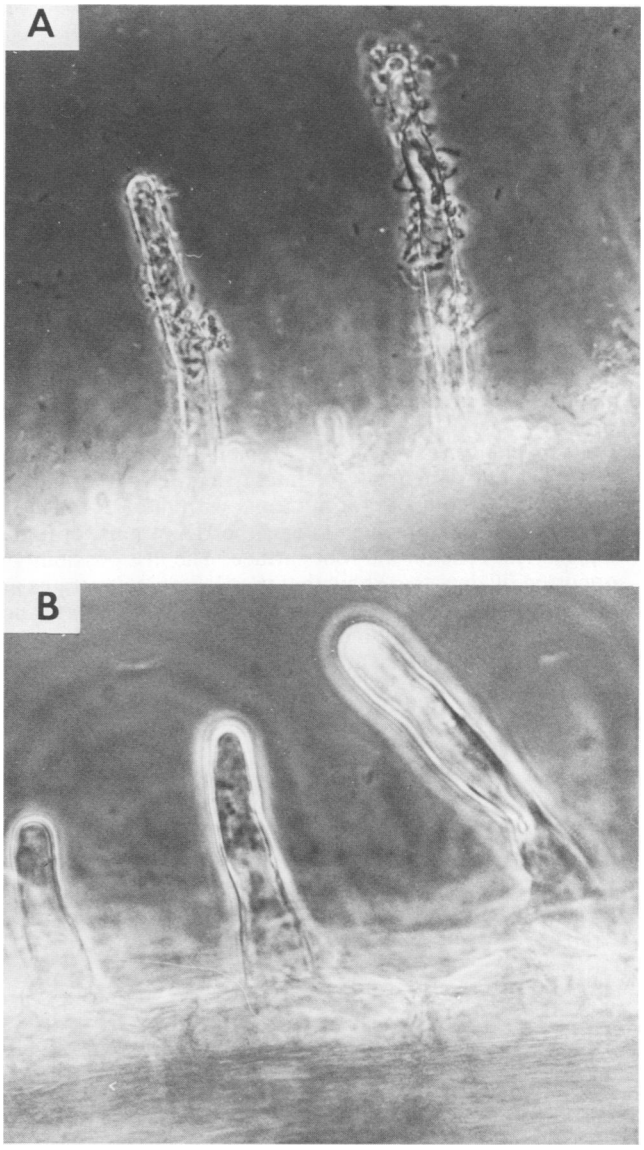

FIG. 1. Treatment of Vernal alfalfa plants with the bacteriocide CGA78039. (A) Plants germinated in sterile distilled water only. Note the bacteria on the root hairs. (B) Plants treated with CGA78039. Note that the root hairs are free of bacteria. Magnification, ×1,600.

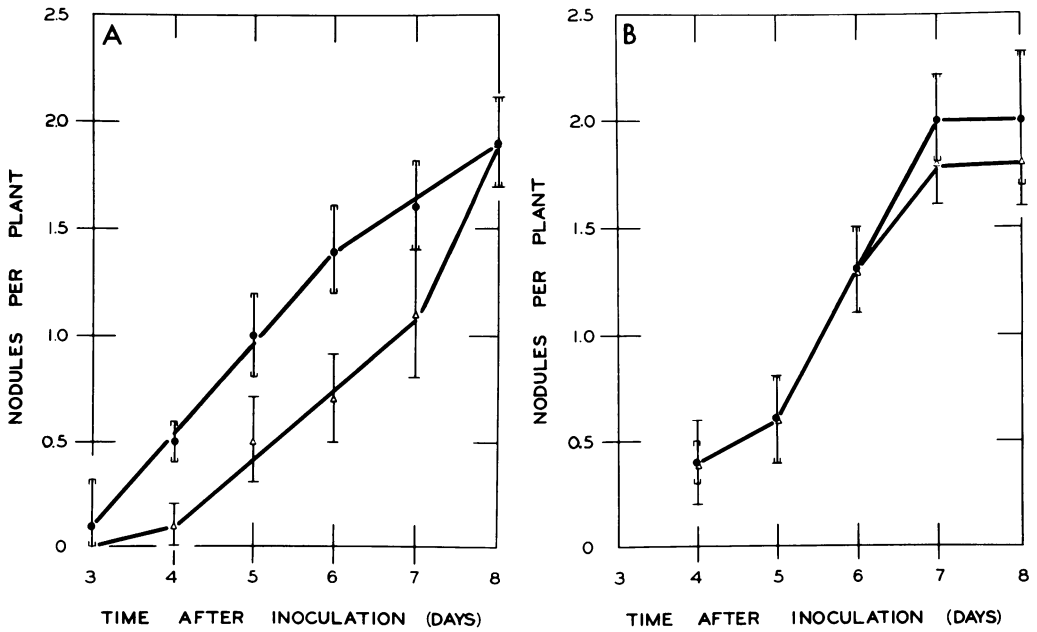

FIG. 2. Nodulation of Vernal alfalfa by *R. meliloti* WL200 and WL100 in the presence and absence of *E. herbicola*. (A) Each plant was inoculated with 10^9 cells of the HA *R. meliloti* strain WL200. (B) Each plant was inoculated with 10^9 cells of the LA *R. meliloti* strain W100. Open triangles represent plants that were inoculated with 10^8 cells of the *E. herbicola* isolate from Vernal alfalfa before the addition of the *R. meliloti*. Closed circles represent plants inoculated only with the *R. meliloti*.

variety of seeds (7), but it has been reported only recently to be in alfalfa seeds (2). The possible presence of *E. herbicola* could interfere with studies such as those on *Rhizobium* binding to seedlings grown from surface-sterilized alfalfa seeds.

The mechanism by which *E. herbicola* inhibits nodulation by the HA strains will be interesting to determine. *E. herbicola* may compete for nutrients with the HA strains, it may produce a toxin which affects the HA strains, or it may displace HA cells on the root surface. The last possibility is supported by data that show that the *E. herbicola* strains are agglutinated by the alfalfa agglutinin at very low titers, such as 1:2 under the conditions described previously (3). If the alfalfa agglutinin is an attachment receptor, *E. herbicola* may compete with *R. meliloti* for these attachment sites. In whatever way the HA cells are affected by *E. herbicola*, they seem to overcome the inhibition and ultimately induce the formation of the same number of nodules as the LA strains. It is possible that the plant induces resistance to the effect of *E. herbicola* in the HA cells and that the LA cells are resistant under all conditions. This would account for the rapid nodulation by the LA cells and the delayed nodulation by the HA cells in the presence of *E. herbicola*.

In a simple laboratory system we have demonstrated the influence of a single alfalfa-associated epiphyte on nodulation by *R. meliloti*. In the soil, in the presence of many organisms, the complexity of the nodulation is much greater. It will be important to identify other biological influences on nodulation to understand the early events in nodulation at the root surface-soil interface. Identification of interactions

TABLE 2. Nodulation by HA and LA strains in the presence and absence of *E. herbicola* from Vernal alfalfa[a]

Inoculum strain	No. of nodules (mean ± SE) 6 days after inoculation:		
	Without *E. herbicola*	With *E. herbicola*	Without/with *E. herbicola*
WL200 (HA)	1.5[b] ± 0.17	0.9 ± 0.16	1.7
WL201 (HA)	2.0[b] ± 0.20	1.6 ± 0.20	1.3
WL218 (HA)	2.0[b] ± 0.27	1.3 ± 0.21	1.5
WL251 (HA)	2.1[b] ± 0.16	1.4 ± 0.19	1.5
WL252 (HA)	1.2[b] ± 0.20	0.4 ± 0.10	3.0
WL100 (LA)	1.8 ± 0.21	2.1 ± 0.20	0.9
WL101 (LA)	1.9 ± 0.27	2.5 ± 0.17	0.8
WL109 (LA)	0.9 ± 0.16	0.8 ± 0.16	1.1
WL120 (LA)	2.2 ± 0.20	2.0 ± 0.20	1.1

[a] All plants were treated with CGA78039 to eliminate the *E. herbicola* before planting. Each value represents the mean number of nodules on 50 plants 6 days after inoculation. Both determinations, with and without *E. herbicola*, for each strain were made in the same experiment.

[b] Significantly more nodules were induced in the absence of *E. herbicola* than in the presence of *E. herbicola* at the 95% confidence level by Student's *t* test.

TABLE 3. Nodulation by strains WL100 and WL200 and Vernal alfalfa in the presence of various *E. herbicola* isolates[a]

Source of *E. herbicola*	No. of nodules (mean ± SE) 6 days after inoculation with:		
	WL100	WL200	WL100/ WL200
	1.8 ± 0.21	2.1 ± 0.26	0.9
Vernal	2.1[b] ± 0.20	1.2 ± 0.17	1.8
Sonora	2.2[b] ± 0.20	1.6[b] ± 0.23	1.4
M. falcata	1.3[b] ± 0.23	0.9[b] ± 0.14	1.4
Saranac	1.5 ± 0.20	1.2 ± 0.18	1.2
Iroquois	1.6 ± 0.18	1.3 ± 0.20	1.2
Hairy Peruvian	0.8 ± 0.22	1.0 ± 0.20	0.8
NC8376	1.6 ± 0.24	1.5 ± 0.21	1.1

[a] All plants were treated with CGA78039 to eliminate the *E. herbicola* before planting. Each value represents the mean number of nodules on 50 plants 6 days after inoculation, except for the values for the Hairy Peruvian isolate, which represents the mean number of nodules on 20 plants.

[b] Denoted is a significant difference at the 95% confidence level by Student's *t* test between the number of nodules induced by strain WL100 and the number of nodules induced by strain WL200.

that affect the time of nodulation and competitiveness of *Rhizobium* strains may help in the production of valuable seed inoculants.

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LITERATURE CITED

1. **Egli, T., and W. Zeller.** 1981. A novel bacteriocide for the control of fireblight. Acta Hortic. **117**:107–111.
2. **Gulash, M., P. Ames, R. C. Larosiliere, and K. Bergman.** 1984. Rhizobia are attracted to localized sites on legume roots. Appl. Environ. Microbiol. **48**:149–152.
3. **Handelsman, J., R. A. Ugalde, and W. J. Brill.** 1984. *Rhizobium meliloti* competitiveness and the alfalfa agglutinin. J. Bacteriol. **157**:703–707.
4. **Holland, A. A., and C. A. Parker.** 1966. Studies on microbial antagonism in the establishment of clover pasture. II. The effect of saprophytic soil fungi upon *Rhizobium trifolii* and the growth of subterranean clover. Plant Soil **25**:329–340.
5. **Lelliott, R. A.** 1974. *Erwinia*, p. 332–340. *In* R. E. Buchanan and N. E. Gibbons (ed.), Bergey's manual of determinative bacteriology, 8th ed. The Williams & Wilkins Co., Baltimore.
6. **Leps, W. T., W. J. Brill, and E. T. Bingham.** 1980. Effect of alfalfa ploidy on nitrogen fixation. Crop Sci. **20**:427–430.
7. **Mundt, J. O., and N. F. Hinkle.** 1976. Bacteria within ovules and seeds. Appl. Environ. Microbiol. **32**:694–698.
8. **Panthier, J. J., H. G. Diem, and T. Dommergues.** 1979. Rapid method to enumberate and isolate soil actinomycetes antagonistic towards rhizobia. Soil Biol. Biochem. **11**:443–445.
9. **Pugashetti, B. K., J. S. Angle, and G. H. Wagner.** 1982. Soil microorganisms antagonistic towards *Rhizobium japonicum*. Soil Biol. Biochem. **14**:45–49.
10. **Ramirez, C., and M. Alexander.** 1980. Evidence suggesting protozoan predation on *Rhizobium* associated with germinating seeds in the rhizosphere of beans (*Phaseolus vulgaris* L.). Appl. Environ. Microbiol. **40**:492–499.