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Method for Testing Degree of Infectivity of *Rhizobium meliloti* Strains

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The infectiveness of different strains of *Rhizobium meliloti* was tested with a technique that uses the addition of tetracycline to the root medium. To stop the infection, the antibiotic was added some time after the inoculation of *Medicago sativa* plants. A coefficient of infectivity for each strain was calculated according to the number of nodules that appeared with and without the addition of the antibiotic. This method seems useful in infectivity studies and is simpler and easier to perform than the test of competence between strains.

Infectiveness is the ability of rhizobia to enter the root cells of the corresponding legume to form the nodule where nitrogen will be fixed. This symbiotic characteristic varies according to the bacterial strain when a defined legume and cultivar is used as the host. Currently, there is no simple method for testing infectivity of different rhizobial strains. The number of nodules formed in the roots of the inoculated plants does not reflect the higher or lower degree of infectivity of the bacterial strain tested, since the number of infection-sensitive sites is always limited (2, 7). Badhuri (2) found that the nodulation rate increased only slightly when plants of *Vigna radiata* grew in hydroponic culture in the presence of 89 to 8.9 $\times$ 10^4 cells ml^{-1}. Bacterial proliferation in the rhizosphere and the limited sites for nodule formation (6, 8) are the causes of such results. Purchase and Nutman (12) used an ingenious method for controlling the multiplication of a virulent *Rhizobium trifolii* strain in the clover rhizosphere: addition of a large inoculum of an avirulent mutant. This allowed them to examine the relation between clover nodule formation and number of virulent bacteria in the rhizosphere. Experiments using competition between strains, employing easily detectable traits (antibiotic resistance or serological identification), have been conducted to test the degree of infectivity of a *Rhizobium* strain. In this paper an alternative method is proposed. It involves the addition of tetracycline to the rooting medium after inoculation, which will inhibit the bacterial cells that have not entered the root cortex.

MATERIALS AND METHODS

Microorganisms. The strains of *Rhizobium meliloti* used in this work are listed in Table 1. Wild-type strain Rm4 was isolated in this laboratory from *Medicago sativa* root nodules. Strain Rm4c was derived from Rm4 after treatment with acridine orange (10). A streptomycin-resistant mutant of Rm4 was obtained by selection on medium containing 1 mg of streptomycin ml^{-1}. Auxotrophic and rough mutants were obtained by treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Sigma Chemical Co., St. Louis, Mo.) as previously described (3). All of the strains used were tetracycline sensitive.

Culture media. Cells were grown on medium 79 of Allen (1). In competence experiments, this medium, supplemented with 1 mg of streptomycin (Sigma) ml^{-1}, and minimal medium of Hooykaas et al. (5) were used.

Nodulation test with different inocula and times of inoculation. Seedlings of *M. sativa* 2 days old (from seeds surface sterilized with 2.5% $HgCl_2$ and germinated on filter paper in petri dishes) were placed on filter paper strips in glass tubes (20 by 200 mm; five plants per tube) containing 10 ml of mineral solution (13). This volume was maintained throughout the experiments by adding sterile water twice weekly. The tubes were placed under controlled conditions of temperature and light (16 h of light at 24 to 26°C and 8 h of dark at 20 to 22°C) and were inoculated, according to the experimental objectives, with suspensions of 48-h cells grown on solid medium. Sylvania Grolux tubes were used as the light source. Nodules were counted 15 to 20 days after inoculation. Bacterial numbers in inocula were determined by plate counts. In some experiments this ranged from 980 to 10^6 cells ml of the seedling medium^{-1} and were added at sowing (10 to 20 tubes in each experiment). In other experiments, 1 ml of inoculum per tube, containing 10^5 cells ml^{-1}, was added either at sowing or after week 1, 2, 3, or 4 (10 to 20 tubes at each time). Rhizobial multiplication was controlled throughout the experiments.

Use of tetracycline to control nodulation. Tubes with plants which were inoculated with a bacterial suspension to reach 10^5 cells ml^{-1} 3 weeks after sowing were supplemented at 0, 24, 48, 72, 96, or 120 h after inoculation with a sterile solution of tetracycline (Sigma). This was added to the plant culture tube to give a final concentration of 10 μg ml^{-1}. Ten to 20 tubes were used for each time period, and a series of inoculated tubes without antibiotic was used as a control. The time (see Table 4) selected to test the

TABLE 1. *Bacterial strains tested*

Strain	Phenotype			Source
	Cultural characteristic	Colonial morphology	Symbiotic ability	
Rm4	Prototrophic	Mucoid	Inf^+ Eff^+	10
Rm4 Str^r	Str^r, prototrophic	Mucoid	Inf^+ Eff^+	This paper
GRC1[a]	Ade^-	Mucoid	Inf^+ Eff^-	This paper
Rm4c	Prototrophic	Semimucoid	Inf^+ Eff^+	10
GRC2[b]	Leu^-	Semimucoid	Inf^+ Eff^-	This paper
GRC4[b]	Phe^- Arg^-	Semimucoid	Inf^+ Eff^+	This paper
GRC5[b]	Lys^-	Semimucoid	Inf^+ Eff^-	This paper
GRC60[b]	Prototrophic	Rough	Inf^+ Eff^+	This paper

[a] Derived from Rm4.
[b] Derived from Rm4c.

degree of infectivity of most of the strains studied was 48 to 72 h. However, some less infective strains may require 96 to 120 h. The coefficient of infectivity (CI) of a strain may be determined by the following formula, which is proposed as an index for infectivity:

$$CI = \frac{N_{Tc}}{N} \times 100 \quad (1)$$

where N_{Tc} is the number of nodules per five plants formed when tetracycline is added and N is the number of nodules per five plants formed in the absence of the antibiotic.

Competition experiments to test degree of infectivity. Wild-type strain Rm4 was inoculated 3 weeks after sowing, as was each strain to be studied. A 1:1 ratio to reach a final concentration of 10^5 cells ml^{-1} in the mineral solution was used. Strain Rm4 has been taken as the type strain for infectivity studies. Two to 3 weeks after inoculation, the 25 to 50 nodules formed by each pair (wild type and mutant) were tested, and the characteristics of the bacterial strains were studied (streptomycin resistance, auxotrophy, or colonial morphology) to establish the ratio of wild-type to mutant strains that formed these nodules. A formula similar to formula 1 may be applied to determine the coefficient of infectivity by this method:

$$CI_c = \frac{n}{N} \times 100 \quad (2)$$

where CI_c is the coefficient in infectivity derived from competence studies, n is the number of nodules formed by the mutant strain, and N is the total number of nodules studied. Nodules in which both wild-type and mutant strains were present, roughly 10%, were not considered.

RESULTS

Table 2 shows the response of the nodulation rate to different bacterial cell densities. There was no significant difference, although the concentration of cells varied 1,000-fold.

The effect of the time of inoculation on the number of nodules formed is shown in Table 3. A maximum number of nodules were formed by delaying inoculation for 3 weeks. Thus, there is no problem in inoculating at this time, when a good root system has developed. The use of the technique proposed here requires that infection occur in a short time; thus, it is necessary that the maximum number of possible infection sites be ready when the plants are inoculated.

TABLE 2. *Effect of inoculum density of strain Rm4 on nodule numbers in alfalfa roots*

Cells/ml	No. of nodules/five plants[a]
980	40.0 ± 5.2
3,910	41.7 ± 7.0
15,620	40.5 ± 6.3
62,500	37.9 ± 5.4
150,000	37.5 ± 4.0
1,000,000	39.3 ± 4.4

[a] Differences were not significant for any of these values.

TABLE 3. *Effect of the time of inoculation with strain Rm4 on nodule numbers*

Inoculation time (wk)	No. of nodules/five plants[a]
0	32.9 ± 6.5[b]
1	28.0 ± 6.0[b]
2	33.8 ± 4.5[b]
3	40.2 ± 0[c]
4	31.0 ± 5.9[b]

[a] Nodules were counted 2 weeks after the last inoculation time.
[b,c] $P \leq 0.05$.

Table 4 shows the effect of the addition of tetracycline at different times on plants inoculated with strains Rm4 and GRC4. Cells that are not inside the roots are affected by tetracycline (as has been demonstrated by taking samples of the tubes after addition of the antibiotic), whereas those that already have entered plant cells continue the infection process. The same results are obtained with rifampin (Sigma), 20 $\mu g\ ml^{-1}$.

Table 5 summarizes the results obtained with

TABLE 4. *Effect of addition of tetracycline on nodule formation by R. meliloti strains Rm4 and GRC4*

Time of addition of tetracycline (h)	No. of nodules/five plants	
	Rm4	GRC4
0	0	0
24	16.6 ± 2.6	1.0 ± 0.3
48	23.1 ± 1.9	2.2 ± 0.8
72	25.1 ± 2.6	6.5 ± 2.0
96	35.1 ± 3.0	17.3 ± 3.2
120	42.5 ± 3.0	25.4 ± 3.8
No addition	42.1 ± 3.0	43.3 ± 1.5

TABLE 5. *Effect of addition of tetracycline 48 h after inoculation on nodule numbers formed by different R. meliloti strains and coefficients of infectivity*[a]

Strain	No. of nodules		CI	CI_c[b]
	Without Tc	With Tc		
Rm4	42.1 ± 3.0	23.1 ± 1.9	54.8	
Rm4 Str^r	42.9 ± 4.0	24.2 ± 1.5	56.4	50
Rm4c	42.0 ± 3.2	15.5 ± 2.4	36.9	37
GRC1	43.0 ± 3.7	13.8 ± 2.0	32.1	35
GRC2	41.7 ± 2.3	10.4 ± 1.5	24.9	23
GRC5	41.9 ± 2.0	6.5 ± 1.5	15.5	14
GRC60	42.0 ± 4.0	4.1 ± 0.3	10.2	12
GRC4	43.3 ± 1.5	2.2 ± 0.8	5.1	5.5

[a] Tc, Tetracycline; CI, coefficient of infectivity (see text).

[b] Strain Rm4 was used as a reference.

all the bacterial strains studied. The coefficient of infectivity obtained in competence studies is also included.

According to the results obtained with this method, all strains derived from the wild type, except the streptomycin-resistant mutant, show a lower virulence. When tetracycline is not added, the nodulation rate is of the same order in all of the strains tested. Resistance to streptomycin does not decrease the level of infectivity of the parent strain.

From these results it can be deduced that each strain studied shows a different degree of virulence, as observed with the technique using addition of tetracycline. The coefficients of infectivity obtained with both methods are almost identical.

DISCUSSION

Although several authors (7, 12) have reported that the number of nodules formed in legume roots is directly related to the bacterial density in the rhizosphere, the results in Table 2 show that *R. meliloti* infecting *M. sativa* behaves in the same way as the bacterium and plant used by Badhuri (2). Under the conditions used in the experiments reported in this paper, the nodulation level was not related to the number of cells present initially in the inocula: at the end of the experiments the number of nodules formed was the same. The results in Table 5 show that several bacterial strains, which in experiments of competence appear to be less competitive, are able to form the same number of nodules when they alone are in contact with roots. To simplify the tedious procedures involved in competence experiments, in which isolation of bacteria from a significant number of nodules must be followed by strain identification, an alternative method may be used. The addition of an antibiotic some time after inoculation stops bacterial proliferation outside the roots. The more rapidly bacteria infect the plant, the less they will be affected by the antibiotic, because they are already well inside the plant cells; this can be deduced from the results obtained when tetracycline is added later after inoculation.

Although infection of the roots can occur throughout the life of the plant (4, 9), it has been necessary to test the nodulation rate of *M. sativa* when the plants have a root system sufficient to obtain the maximal infection. Three weeks after sowing has been selected as the best time to inoculate the plants (see Table 3), and 48 or 72 h is the optimal time to add the antibiotic (see Table 4). Under these conditions, differences in the number of nodules formed in the sets of plants with and without antibiotic were obtained. However, in cases of very low infectivity it might be better to wait until 96 or 120 h after inoculation to add the antibiotic in order to detect infection.

Relatively small inocula were used to avoid the possibility of selecting tetracycline-resistant mutants (in the strains used, the frequency of tetracycline-resistant mutations was lower than 10^{-7}) and to minimize the effect on bacterial substances involved in the infection process. These substances could be concentrated enough when large inocula of a less infective strain are used.

Although several antibiotics were assayed (streptomycin, ampicillin, rifampin, and kanamycin), tetracycline was selected because the others, save rifampin, either had no effect on bacteria or seriously affected the plants. Although, according to Peterson and Sinha (11), tetracycline could enter the plant cells, its action on the different strains would be the same as in culture media. Use of strains carrying plasmid R68.45 has revealed that the inhibitory effects of tetracycline are directed to the rhizobia and not to the plant (J. Casadesús, Ph.D. thesis,

University of Granada, Granada, Spain, 1979). Rifampin, in spite of its suitability, was not extensively used because it is more expensive than tetracycline.

We think the technique presented here is simpler and easier to perform than competence studies. The values obtained with both methods are the same. Only small differences exist between the two coefficients of infectivity presented in Table 5. We think that the percentage of nodules of mixed bacteria, which was the same as that reported by Skrdleta (14), does not affect the results substantially.

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