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Root Surface Association in Relation to Nodulation of *Medicago sativa*

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Nine strains of *Rhizobium meliloti*, ranging in competitive ability on *Medicago sativa* from excellent to poor in autoclaved soils, were paired in 29 combinations and used to inoculate *M. sativa* in a liquid rooting medium. A positive correlation ($r = 0.545$) between strain ratios in nodules after 28 days and root surface cell ratios after 7 days was determined. Two cell fractions from the root surface, representing loosely and firmly adhering cells, were investigated. Infectivity was linked to the more firmly adhering cells. A significant relationship was established between the cell ratios of competing strains in the two fractions. In another experiment, adherence of cells of both infective and noninfective *Rhizobium* strains to roots of *M. sativa* and *Trifolium repens* was demonstrated; the ratios of loosely to firmly adhering cells on the root surface were significantly narrower with the infective combinations than with noninfective strain-legume associations.

Although competitive abilities of strains of *Rhizobium* have received attention in various laboratories (5), quantitative aspects of paired strains have been studied most intensively by Vincent et al. (9, 12, 13). By expressing strain ratios in the inoculum, on the root surface, and in nodules as a quantitative function, they have been able to show that inoculum strain ratios are not necessarily reflected in root surface ratios. A dependence on root surface representation for nodulation success when *Rhizobium* strains are equally influenced by the host and an independence when the host had an overriding influence have been indicated.

To relate nodulation success to root surface cell ratios, Vincent and co-workers (9, 12, 13) determined cell ratios on the root at appropriate intervals to investigate the possibility of different adherence of competing strains with time. It was concluded that with temperate legumes, including *Medicago sativa*, either 3- or 7-day estimates appeared suitable for assessing nodulating competitiveness (9).

While we were determining the competitive abilities in soil of each of 24 strains of *R. meliloti* (11), 9 of the strains were paired concurrently in 29 combinations and used to inoculate seedlings of *M. sativa* in a liquid medium. These strains were compatible with and highly effective on *M. sativa*, and a marked differential host effect (5) on strains was unlikely. By involving such an appreciable number of paired strain combinations, the results were anticipated to yield acceptable information on the reliability of assessing nodulating competitiveness on the basis of a single determination of cell ratios on the root surface.

An additional objective of this study was to compare the degree of attachment of rhizobia with the root surface of infective and noninfective *Rhizobium*-legume combinations.

MATERIALS AND METHODS

***Rhizobium* strains used and preparation of inoculum.** The strains of *R. meliloti* and their streptomycin-resistant (Str^+) mutants used in competition studies are described in the accompanying paper (11). *R. trifolii* SR2 and SR4, highly effective on *Trifolium repens*, were obtained from the South African Rhizobium Collection. Inoculum of a strain was prepared from a yeast-mannitol broth (1) culture cultivated at 27°C. Cells of a 6-day-old culture were harvested and washed by repeated centrifugation and resuspension in sterile water and standardized to ca. 6.0×10^7 to 7.0×10^7 cells ml^{-1}. In competition experiments, the inoculum of each of two strains was serially diluted and mixed 1:1 to provide ca. 650 cells ml^{-1}.

Removal of *Rhizobium* cells from the root surface. At the outset, 7-day-old root systems of infective and noninfective *Rhizobium*-legume combinations, as represented by homologous *R. meliloti-M. sativa* and *R. trifolii-T. repens* combinations and the heterologous *R. meliloti-T. repens* and *R. trifolii-M. sativa* combinations, were shaken by hand 100 times in 10-ml quantities of sterile water in McCartney bottles. Roots were then examined by scanning electron microscopy to determine whether all cells were removed from the surface. The presence of large numbers of polarly attached cells still adhering to the roots required an additional washing process in which roots were shaken 100 times in 10 ml of sterile water containing 3 g of finely sieved marble chips (Endecotts mesh no. 14). Electron microscopic examination confirmed that all

TABLE 1. Adherence of rhizobial cells to the root surface of each of two legume species inoculated with *R. meliloti* or *R. trifolii*

Inoculum	Log no. of rhizobial cells[a] washed from the root surface[b] of:					
	M. sativa			*T. repens*		
	Fraction 1	Fraction 2	1:2 ratio	Fraction 1	Fraction 2	1:2 ratio
R. trifolii SR2	6.1553	5.0645[c]	12.3	6.2430	5.7604	3.0
R. trifolii SR4	6.5276	5.2625[c]	18.4	6.1790	5.9479	1.7
R. meliloti RF4	6.3118	6.0934	1.7	6.2014	5.4487[c]	5.7
R. meliloti RF6	6.1492	5.7917	2.3	5.6628	4.8096[c]	7.1

[a] Average of three replicates.

[b] Cells were released by shaking roots in sterile water (fraction 1) and subsequently in sterile water containing marble chips (fraction 2).

[c] Cell representation in fractions 1 and 2 of a noninfective combination differed significantly from the expected cell representation of an infective combination (χ^2 test) as calculated from average values derived from the infective homologous combinations (16).

cells were removed by the second treatment. Viable counts of cells in the water used in the two washing processes were designated root surface 1 and root surface 2 counts.

Competition experiments. Surface-sterilized seeds of *M. sativa* cultivar Karoo were germinated on yeast-mannitol agar. A selected seedling was transferred aseptically to a glass tube (150 by 20 mm) containing 20 g of glass beads (3-mm diameter) and 5 ml of nitrogen-free Hoagland nutrient solution (10). Each seedling received 0.4 ml of mixed inoculum. Immediately after inoculation, the numbers of wild-type and Str^+ cells in the inoculum were determined on yeast-mannitol agar plates with and without 250 μg of streptomycin per ml.

After 7 days in a greenhouse with temperatures ranging from 8 to 24°C, three replicate plants were removed to determine the numbers of *Rhizobium* cells of each strain of a pair on the root surface. Counts of viable cells in the water used in the two washing processes were made on yeast-mannitol agar with and without 250 μg of streptomycin per ml; 21 days later, another three replicates of each treatment were used for the isolation and identification of strains in nodules (11).

Ratios of Str^+ mutant to wild-type strains in the inoculum, on the root surface, and in nodules were calculated and expressed as X:1. Significant differences from expected 1:1 ratios were calculated with the χ^2 test (17). Nodule representation of strains was correlated with cell ratios in the inoculum and in the root surface 1 and 2 fractions. The sign test (17) was used to determine the relationship between the cell ratios in the two root surface fractions as follows. When the value of X in a root surface 2 cell ratio was bigger than the value of X in the corresponding root surface 1 cell ratio, it was given a positive (+) sign; a negative (−) sign was given to X in root surface 1. A lower value of X in root surface 2 was given a negative sign, with a plus sign given to the corresponding higher value of X in root surface 1. The significance of these positive or negative signs was calculated against two criteria: i) values of X in nodule ratios significantly <1, and (ii) values of X in nodule ratios ≥1.

Speed of infection. The ability of a strain of *R. meliloti* to infect *M. sativa* rapidly was tested by adding tetracycline to the rooting medium 48 h after seedlings were inoculated. The experimental procedure of Olivares et al. (14) was followed, except that 48-h-old seedlings instead of 21-day-old plants were inoculated.

RESULTS

The degree of attachment to roots of infective (*R. meliloti-M. sativa* and *R. trifolii-T. repens*) and noninfective (*R. meliloti-T. repens* and *R. trifolii-M. sativa*) rhizobia is shown in Table 1. Root surface 1 and root surface 2 cells represent, respectively, free or loosely adsorbed cells and cells firmly attached to the root. It is clear that appreciable numbers of cells of both infective and noninfective rhizobia became firmly attached to the root surface. However, root surface 1-root surface 2 cell ratios in the case of the infective combinations were significantly narrower than those of the noninfective *R. meliloti-T. repens* and *R. trifolii-M. sativa* combinations (Table 1).

The results obtained in the competition experiment with nine strains of *R. meliloti* paired in 29 combinations (Table 2) show the following. (i) A highly significant correlation ($r = 0.545$) existed between the ratio of strains in nodules after 28 days and the ratio of cells of these strains firmly attached to the root surface (root surface 2) after 7 days (Table 2). (ii) No correlation was found between the ratio of strains in nodules and the ratio of cells of these strains in either the inoculum or the root surface 1 fraction (Table 2). (iii) If values of X of <1 or ≥1 in nodule ratios are taken as criteria, a highly significant relationship between the cell ratios in the root surface 1 and 2 fractions is apparent (Table 2). (iv) Strain RF24, which was indicated as the most competitive strain for nodulation in experiments conducted in autoclaved soils (11), was among the weak competitors in this study.

In a final experiment, we attempted to determine whether the strains of *R. meliloti* identified as good competitors also infected roots rapidly.

TABLE 2. Ratio of strains of *R. meliloti* applied in pairs as mixed inocula to *M. sativa* in the inoculum, on the root surface, and in nodules[a]

Strain pair (Wild type × Str$^+$ mutant)	Value of X in Str$^+$ mutant A–wild-type ratios (X:1)				
	Inoculum	Root surface[b]		X bigger (+) or smaller (−) in 2 than in 1[c]	Nodules
		Fraction 1	Fraction 2		
RF6 × RF4	0.99	0.30	1.04	+	1.05
× RF24	1.64	0.29	0.11	−	0.33[d]
× RF8	1.32	0.59	0.48	−	0.48[d]
× RF23	2.62	0.28	0.50	+	0.71
× RF5	1.03	0.32	0.37	+	0.67
× RF6	0.78	0.49	1.31	+	1.40[e]
× RF15	1.41	1.52	0.40	−	0.22[d]
× RF22	0.97	1.58	0.27	−	0.08[d]
× RF14	1.06	0.15	0.75	+	0.44[d]
RF4 × RF6	0.80	0.41	0.91	+	1.03
× RF24	0.96	2.45	0.38	−	0.06[d]
× RF8	0.83	0.15	0.10	−	0.33[d]
× RF23	1.32	0.31	0.83	+	0.64
× RF5	1.07	0.20	0.17	−	0.50[d]
× RF4	1.14	1.67	0.87	−	2.10[e]
× RF15	1.78	1.15	0.28	−	0.25[d]
× RF22	1.21	0.43	0.21	−	0.45[d]
RF24 × RF4	1.06	0.70	0.79	+	7.00[e]
× RF6	0.66	0.15	0.94	+	1.57[e]
× RF8	1.95	0.49	0.12	−	0.71
× RF23	1.27	0.16	0.42	+	0.80
× RF5	1.84	0.81	0.48	−	0.11[d]
× RF24	1.27	0.47	0.95	+	1.20
RF15 × RF6	0.97	0.63	2.11	+	5.50[e]
RF15 × RF4	0.88	0.16	5.13	+	4.00[e]
RF22 × RF6	1.28	0.62	1.00	+	2.66[e]
RF22 × RF4	1.97	0.32	2.40	+	4.00[e]
RF14 × RF6	0.76	0.41	2.00	+	11.00[e]
RF14 × RF4	0.95	0.18	2.22	+	1.50[e]

[a] Average of three replicates. Cell or strain ratios in inoculum, on the root surface, and in nodules were determined, respectively, at the time of inoculation, after 7 days, and after 28 days. The correlation between the ratio of strains in nodules after 28 days and the ratio of strains attached to root surface 2 was highly significant ($r = 0.545$); $t = 4.364$; $t(0.05) = 2.045$; $t(0.01) = 2.756$. The root surface 1 cell ratio-nodule ratio correlation and the inoculum cell ratio-nodule ratio correlation were not significant ($r = 0.17$ and 0.22, respectively).

[b] Cells were released by shaking roots in sterile water (root surface 1) and subsequently in sterile water containing marble chips (root surface 2).

[c] If a nodule ratio in which X is significantly smaller than 1 is taken as the criterion, the possibility is only 0.12 that a smaller value of X in the root surface 2 ratio than that in the root surface 1 ratio was due to chance. If a nodule ratio in which X is greater than or equal to 1 is taken as the criterion, the possibility is only 0.004 that a bigger value of X in root surface 2 was due to chance.

[d] X significantly smaller than 1.

[e] X, >1.

Tetracycline was added to inoculated seedlings 48 h after inoculation to kill off the sensitive rhizobia which had not at that stage entered the root hairs. In general, competitive ability as determined in autoclaved soils (11) correlated with the ability to infect plants rapidly; for example, strains RF4, RF6, and RF24, which were included because of their excellent competitive abilities, were also the most rapid to infect the seedlings (Table 3).

TABLE 3. Nodules formed by *R. meliloti* on *M. sativa* when tetracycline was added to the rooting medium 48 h after inoculation

R. meliloti strain	Nodules formed (%)[a]
RF24	82.14
RF6	77.78
RF4	45.16
RF15	40.91
RF14	37.50
RF5	37.04
RF22	17.39

[a] Expressed as percentage of nodules formed by a strain in the absence of the antibiotic. Each treatment consisted of seven plants.

DISCUSSION

The experimental procedure followed in this investigation differed from that of Vincent and collaborators (9, 12, 13) in several ways, notably with regard to inoculum density, growth medium, and the fact that cells on the root surface were divided into two fractions. Nevertheless, the significant positive correlation between the ratios of paired strains in nodules and the ratios of cells of these strains firmly adhering to the root surface agreed in general with the findings of Vincent et al. (9, 12, 13).

In this study, a very light inoculum consisting of competing strains was used to allow a full opportunity for each strain to express its growth pattern. Although possible drifts in the root surface representation of strains with time had to be considered, it appeared from the results of Vincent et al. (9) that with paired strains compatible with the host, changes in root surface cell ratios with time were minimal when the inoculum ratio was close to 1. Because of the large number of strain combinations used, the significant positive correlation established between ratios of paired strains in nodules and their cell ratios on the root after 7 days could hardly be interpreted as fortuitous. It was considered confirmation that a reliable indication of nodulating competitiveness of paired strains could be derived from a single determination of root surface cell ratios.

It might be argued that the approach for differentiating between loosely and firmly adhering cells on the root surface was of little significance, as the mechanism responsible for this phenomenon has not been investigated. However, there were strong indications that the positive correlation between nodule representation and the cell ratios in fraction 2, but not in fraction 1, did not simply reflect the presence in fraction 1 of a significant proportion of nonadhering cells from the rooting medium. First, cell representation in the two fractions from noninfective *Rhizobium*-legume combinations differed significantly from that calculated for infective combinations, suggesting a definite relationship between the two cell fractions (Table 1). Second, a highly significant relationship was established between the cell ratios of the root surface 1 and 2 fractions of competing strains which linked infectivity to the more tightly adhering cells (Table 2). Somewhat puzzling was the fact that other investigators apparently had no problems in washing adhering cells from roots by shaking alone, whereas we had to employ marble chips to remove the cells comprising fraction 2.

Since Bohlool and Schmidt (3) indicated a specific reaction between strains of *R. japonicum* and soybean lectin, many reports have linked specificity in the *Rhizobium*-legume symbiosis to a specific recognition interaction between infective bacteria and root hairs (4, 6). Attempts have been made to determine the exact region on the root where infection takes place (2), and it has been stressed that studies aimed at correlating aspects of infectivity with cell attachment to the root surface should deal with those cells adsorbed to the specific zone where infection takes place (15).

Adsorption of rhizobia to legume root surfaces is common to both infective and noninfective *Rhizobium*-legume combinations (8), as was also demonstrated in this study with homologous and heterologous strain-legume combinations (Table 1). Therefore, to explain specificity in terms of the suggested early recognition interaction between infective bacteria and root hairs before infection (6, 7), a specific mechanism which allows bacteria to attach to roots in high numbers and a nonspecific mechanism which allows attachment in lower numbers were proposed (8).

With highly compatible strain-host combinations in which selective host effects on strains are unlikely and in which cells of competing strains are present in approximately equal numbers in the rooting medium, adherence to the root surface of nonspecifically adsorbed cells would probably occur in a random fashion to give a cell ratio of ca. 1:1. Deviations from this ratio would presumably reflect, although in a somewhat masked way, ratios of specifically adsorbed infective cells. This assumption was supported by the positive correlation obtained between strain ratios in nodules and their cell ratios as determined over the entire root surface.

The competitive ability of a strain is often associated with its ability to infect rapidly (14), as also indicated by the results of this study. Strains RF4, RF6, and RF24, which were identified as the best competitors on *M. sativa* in soil (11), were the most rapid to infect; weak com-

petitors such as RF14, RF15, and RF22 were all relatively slow to infect. The inability of strain RF24, the most outstanding strain in competition experiments in autoclaved soils, to compete successfully in the liquid growth medium used in this study cannot be explained at this stage and warrants further attention.

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