Plant and Soil 82, 223–229 (1984). Ms. 5812

Effect of heterologous bacteria and combined nitrogen on the adsorption of *Rhizobium meliloti* to lucerne seedling roots*

CAROLE LAFRENIERE, L. M. BORDELEAU,
Station de recherches, Agriculture Canada, 2560 boulevard Hochelaga, Sainte-Foy, Québec, Canada G1V 2J3

N. AMARGER
Institut National de la Recherche Agronomique, Laboratoire de Microbiologie des Sols, B. V. 1450, 21034 Dijon Cedex, France

and H. ANTOUN
Département des sols, FSAA, Université Laval, Québec, Canada G1K 7P4

Received 10 February 1984. Revised June 1984

Key words *Agrobacterium tumefaciens* Combined nitrogen Lectin recognition *Medicago sativa* Radioactive bacteria *Rhizobium meliloti* *Rhizobium trifolii* Seedling roots

Summary The binding of *Rhizobium meliloti* strains A_2 (effective) and V_6 (ineffective), *Agrobacterium tumefaciens* strain B_6S_3 and *R. trifolii* strain TL_5 to lucerne seedling roots was studied by using ^{14}C or ^{3}H-labelled bacteria. When added singly or in combination with the heterologous bacteria, the number of A_2 cells attached to the roots was significantly less than the number of B_6S_3 or TL_5 cells. However, the presence of the heterologous bacteria did not decrease the proportion of A_2 cells added in the inoculum that bind to the roots, suggesting that *R. meliloti* is attached to specific sites. In fact, the same number of A_2 or V_6 cells bind to the roots and in mixed inoculation the 2 strains share equally the binding sites. When added to the seedlings growth medium NO_3^- at 5 or 16 mM significantly decreased the number of A_2 cells adhering to lucerne seedling roots. The results suggest that the lectin-recognition hypothesis is probably involved in the attachment of *R. meliloti* to lucerne seedling roots.

Introduction

In the Rhizobium-legume symbiosis, the formation of nodules is the result of a complex multi-step process in which rhizobial attachment to legume root hairs is an early step and may be involved in the control of specificity[4]. In fact, observations suggest that recognition at infection sites involves the binding of specific legume lectins to unique carbohydrates found exclusively on the surface of the appropriate rhizobial symbiont[8].

An agglutinin responsible for specific recognition between *Rhizobium meliloti* and lucerne seedling roots, was isolated from lucerne

* Contribution No. 252 Station de recherches, Agriculture Canada

seeds and detected on the roots[13]. The present work represents the results of experiments designed to determine whether *R. meliloti* exhibit a preferential or specific attachment to lucerne seedling roots. As combined nitrogen is one of the many environmental factors which limits the development and success of the Rhizobium-legume symbiosis in nature and can regulate rhizobial adsorption to host root hairs[7] and root-hair infection[12], the effect of NO_3^- and NH_4^+ on the adsorption of *R. meliloti* to lucerne seedling roots was also investigated.

Materials and methods

Bacteria

R. meliloti strains A_2 (effective) and V_6 (ineffective)[3], *R. trifolii* strain TL_5 and *Agrobacterium tumefaciens* strain B_6S_3 were used. Strain B_6S_3 was obtained through the courtesy of Dr. P. Dion, Département de phytologie, Université Laval.

Growth of seedlings

Lucerne seedlings were grown by using the Petri dish method[10] modified as follows. Seeds of the cultivar Saranac of lucerne (*Medicago sativa* L.) were surface sterilized[15] and germinated on sterile agar (1%) for 18 h in the dark. Ten uniform germinated seeds were transferred to midline of Petri plates containing a solid (1.5% agar) nitrogen free nutrient solution (ingredients in mg per litre of distilled water): $MgSO_4.7H_2O$, 492.96; K_2HPO_4, 174.18; KH_2PO_4, 136.09; $CaCl_2$, 110.99; ferric citrate (0.5%), 5; H_3BO_3, 2.86; $MnCl_2.4H_2O$, 1.81; $ZnSO_4.7H_2O$ 0.22; $CuSO_4.5H_2O$, 0.08; $H_2MoO_4.H_2O$, 0.09; $CoCl_2.6H_2O$, 0.004; pH, 6.8. The plates were partially sealed with Parafilm (American Can Co., Greenwich) and placed in a vertical position in a growth chamber under a 16 h light period ($360\ \mu E.m^{-2}.s^{-1}$) at 18–20°C and 8 h darkness at 13–15°C.

Quantitative assays for adsorption of bacteria to lucerne seedling roots

Rhizobia were cultured on yeast extract mannitol broth[15] and *A. tumefaciens* on Difco Bacto-nutrient broth. Fifty ml portions of the culture media were inoculated with the appropriate bacteria and 1.85×10^6 Bq of D-[U-^{14}C] glucose or 9.25×10^5 Bq of D-[6-^{3}H(N)] glucose (NEN, Montréal, Canada) was added to the culture in 250 ml Erlenmeyer flasks. The flasks were incubated at 30°C on a rotary shaker operating at 160 rpm. Cells were collected in late log phase and washed 3 times in 50 ml phosphate buffer saline (PBS; 3 m*M* phosphate buffer in 0.7% NaCl, pH 6.8) by centrifugation (12000*g* for 15 min). The washed bacterial pellet was resuspended in 50 ml PBS and the number of labelled bacteria was determined by plate count and radioactivity was assayed. Ten uniform (4 to 5 days old) lucerne seedlings were transferred to a Petri plate and mixed with 20 ml of PBS containing the labelled bacteria singly or in combination at a final concentration of about 1 to 3×10^8/ml. The plates were incubated for 1 to 4 h with gentle occasional shaking. The seedlings were washed in PBS for 15 min and the roots were then excised and added to 1 ml Protosol (NEN, Montréal, Canada) in scintillation vials. The vials were capped and allowed to stand at 25°C for 24 h and 10 ml of Bray's cocktail was added and the pH adjusted to 7 with glacial acetic acid. Radioactivity was counted in a Rackbeta 1217 (LKB/Wallac) liquid scintillation counter. To account for radioactivity taken up from solution rather than in the form of radioactive bacteria[11], a suspension of bacteria in PBS incubated for 4 h was passed through 0.2 μm membrane filter (Nalgene Co., Rochester, N.Y.) and the bacterial filtrate was assayed as described earlier for bacterial suspensions. The mean radioactive count for roots immersed in the bacterial filtrate was substracted from the mean value of radioactivity of the roots immersed in the bacterial suspension, and the difference was divided by the mean value of the

radioactivity of a known number of bacterial cells, thus giving the number of adsorbed cells per 10 seedling roots.

Effect of combined nitrogen on adsorption of R. meliloti *to lucerne seedling roots*

To study the effect of combined nitrogen on the adsorption of *R. meliloti* on roots, lucerne seedlings were grown as described earlier on solid nitrogen-free nutrient solution or on the nutrient solution supplemented with different concentrations of NO_3^- (as KNO_3), NH_4^+ (as NH_4CL) or both ions (as NH_4NO_3). The attachment of strain A_2 ^{3}H-*R. meliloti* was assessed as described earlier: in PBS for seedlings grown in the presence of combined nitrogen or in PBS supplemented with the different concentrations of combined nitrogen for seedlings grown on nitrogen-free nutrient solution.

Statistical analysis.

In all assays the Petri dishes used to produce lucerne seedlings were placed in the growth chamber according to a randomized complete block design. Results were compared by using Duncan's multiple range test or Dunnett test on natural logarithm transformed data[14].

Results and discussion

The binding of strains A_2 of *R. meliloti* and B_6S_3 of *A. tumefaciens* to lucerne seedling roots increased significantly with time (Table 1). However no significant difference was observed in the binding of strain TL_5 of *R. trifolii* when incubated with lucerne seedling roots for more than 1 h. Table 1 shows for strains A_2 and B_6S_3 that the reproducibility of the results increased when bacteria were allowed to interact with roots for more than 1 h. For example in experiment I the coefficients of variation observed with strain A_2 after 1, 2 and 4 h interaction with roots were 37, 16 and 11% respectively. This indicates that under the experimental conditions used, reproducible results for bacterial attachment studies can be obtained if bacteria are allowed to interact with lucerne roots for 2 to 4 h. Strain A_2 of *R. meliloti* was not preferentially adsorbed on lucerne seedling roots. In fact, in all assays performed strain A_2 was adsorbed significantly less than *A. tumefaciens* or *R. trifolii* (Table 1).

In mixed inoculation trials, *A. tumafaciens* and *R. trifolii* were also adsorbed in greater number than *R. meliloti,* but the presence of the heterologous bacteria did not decrease the proportion of *R. meliloti* cells added in the inoculum that bind to lucerne roots (Table 2). This proportion probably represents the rhizobial cells having their lectin receptor carbohydrate[8] completely developed or unaffected. Similar tendencies were observed with another effective *R. meliloti* strain S_{14} (results not shown). These observations might suggest that *R. meliloti* cells are adsorbed on very specific binding sites and that the presence of other non-antagonistic bacteria will not have an adverse effect on lucerne nodulation. In fact, no decrease in lucerne dry matter yield was observed when heterologous rhizobia were added with strain A_2 of *R. meliloti* in the inoculum[2].

Table 1. Time course changes in the adsorption of strains A_2 ^{14}C-*R. meliloti*, B_6S_3 ^{3}H-*A. tumefaciens* and TL_5 ^{3}H-*R. trifolii* on lucerne seedling roots

Time (h) after inoculation	Number of cells adhering per seedling root ($\times 10^5$)*		
	A_2	B_6S_3	TL_5
Experiment I			
1	0.91 ± 0.34 (2.0)**	3.11 ± 0.41 (7.4)	–
2	1.36 ± 0.22 (3.0)	6.25 ± 0.53 (14.9)	–
4	2.46 ± 0.26 (5.3)	9.03 ± 0.35 (21.5)	–
Experiment II			
1	0.75 ± 0.30 (1.4)	–	4.62 ± 0.30 (10.0) NS†
2	1.18 ± 0.26 (2.2)	–	6.03 ± 0.35 (13.1) NS
4	2.08 ± 0.36 (3.4)	–	6.42 ± 0.33 (14.0) NS

* Means ± standard deviation from 10 replicates (100 seedlings).
** Numbers in parentheses indicate the percentage of added cells adhering per seedling root ($\times 10^3$).
† NS, means not significantly different ($P \leq 0.05$) according to Duncan's multiple range test.

Table 2. Adsorption of strains A_2 ^{14}C-*R. meliloti*, B_6S_3 ^{3}H-*A. tumefaciens* and TL_5 ^{3}H-*R. trifolii* on lucerne seedling roots, in mixed inoculation studies

Strains in the inoculum	Number of cells adhering per seedling root ($\times 10^5$) 4 h after inoculation*		
	A_2	B_6S_3	TL_5
Experiment I			
A_2	2.43 ± 0.22 (6)**	–	–
B_6S_3	–	19.47 ± 0.32 (40)	–
$A_2 + B_6$ S3†	1.18 ± 0.17 (6)	7.95 ± 0.33 (33)	–
Experiment II			
A_2	1.27 ± 0.26 (4)	–	–
TL_5	–	–	6.20 ± 0.37 (19)
$A_2 + TL_5$ †	0.73 ± 0.32 (5)	–	2.31 ± 0.48 (15)

* Means ± standard deviation from 7 replicates (70 seedlings). In each experiment means are significantly different ($P \leq 0.05$) according to Duncan's multiple range test.
** Numbers in parentheses indicate the percentage of added cells adhering per seedling root ($\times 10^3$).
† In combination, for each strain the number of cells added was half the number used with single strains.

If this hypothesis is valid, then *R. meliloti* strains will bind in same number on lucerne seedling roots and when combined 2 strains will either compete for the specific binding sites or share them equally. There was no significant difference in the number of cells of the effective strain A_2 and the ineffective nodulating strain V_6 of *R. meliloti* attached to lucerne seedling roots (Table 3), indicating that these strains are adsorbed on the same binding sites. In mixed inoculation, no significant difference was observed between the number of cells of strain A_2 or V_6 adhering to seedling roots (Table 3) indicating

Table 3. Competitive adsorption of *R. meliloti* strains A_2 (^{14}C) and V_6 (^{3}H) on lucerne seedling roots

Strains in the inoculum	Number of cells adhering per seedling root ($\times 10^4$) 2 h after inoculation*	
	A_2	V_6
A_2	4.81 ± 0.33 a (1.5)**	–
V_6	–	5.81 ± 0.26 a (1.8)
$A_2 + V_6$†	3.03 ± 0.10 b (1.9)	3.32 ± 0.20 b (2.1)

* Means ± standard deviations from 7 replicates (70 seedlings).
** Means followed by the same letter are not significantly different ($P \leq 0.05$) according to Duncan's multiple range test. Number in parentheses indicate the percentage of added cells adhering per seedling root ($\times 10^3$).
† In combination, for each strain the number of cells added was half the number used with single strains.

Table 4. Effect of combined N on the adsorption of strain A_2 ^{3}H-*R. meliloti* on lucerne seedling roots. Combined N was added to the seedling growth medium (SGM) and binding assay performed in PBS, or only to PBS (B) for seedlings grown in N-free medium.

Salt supplement	Concentration (m*M*)	Number of cells adhering per seedling root ($\times 10^4$) 4 h after inoculation†	
		SGM	B
Check	–	13.20 ± 0.34	11.02 ± 0.39
KNO_3	5	10.47 ± 0.44*	12.68 ± 0.34
	16	9.49 ± 0.38**	10.91 ± 0.38
NH_4Cl	0.25	13.87 ± 0.28	13.46 ± 0.33
	1	13.46 ± 0.46	12.06 ± 0.43
NH_4NO_3	0.25	12.30 ± 0.32	12.43 ± 0.36
	2	11.13 ± 0.35	13.20 ± 0.42

† Means ± standard deviation from 3 replicates (30 seedlings).
* Significantly less than the check at $P \leq 0.05$, and ** at $P \leq 0.01$ according to Dunnett's test.

that the 2 strains share equally the specific binding sites on roots and will probably form the same number of nodules. In general, when effective and ineffective *R. meliloti* strains were mixed in approximately equal ratios the number of effective nodules formed is very close to the number of ineffective nodules[1].

In the Rhizobium-clover symbiosis, the immunologically detectable levels of trifolin A on the root surface and the specific attachment of *R. trifolii* to root hairs decreased in a parallel fashion as the concentration of either NO_3^- or NH_4^+ were increased in the rooting medium[7]. We further investigated the effect of NO_3^- and NH_4^+ on the adsorption of *R. meliloti* to lucerne seedling roots. The number of cells of the strain A_2 binding to seedling roots was significantly reduced with

plants grown in the presence of 5 or 16 m*M* NO_3^- (Table 4). No significant effect was observed with plants grown in the presence of NH_4^+ or when fixed nitrogen was only added to the buffer (PBS) during the 4 h binding assay. As in *R. trifolii*, NO_3^- probably does not interact directly with lucerne lectin[9], but rather might regulate its synthesis or operate by another unknown mechanism. The absence of an inhibitory effect of NH_4^+ on rhizobial attachment, as observed with clover, can be attributed to different nitrogen metabolism in lucerne and could indicate that the NH_4^+ concentrations used are low.

The present work shows by an indirect method that the lectin-recognition hypothesis[8] is probably involved in the attachment of *R. meliloti* to lucerne seedling roots. This work also illustrates as previously reported[5,6,11], that radioactive bacteria can be useful tools to study the binding of rhizobia to legume roots.

Acknowledgements We thank the Natural Science and Engineering Research Council of Canada, and the Québec ministry of Education (FCAC) for financial support given to C Lafrenière. Part of this work was performed while N Amarger was supported by a fellowship (for nitrogen fixation programme in L M Bordeleau laboratory) from the Organization of Economic Co-operation and Development (OECD).

References

1 Amarger N 1981 Competition for nodule formation between effective and ineffective strains of *Rhizobium meliloti*. Soil Biol. Biochem 13, 475–480.

2 Antoun H, Bordeleau L M and Lachance R A 1979 Rendement de la luzerne (cultivar Saranac) inoculée avec une souche très efficace de *Rhizobium meliloti* en présence d' autres espèces de *Rhizobium*. Can. J. Plant Sci. 59, 521–523.

3 Antoun H, Bordeleau L M and Sauvageau R 1984 Utilization of the tricarboxylic acid cycle intermediates and symbiotic effectiveness in *Rhizobium meliloti*. Plant and Soil, 77, 29–38.

4 Broughton W J 1978 Control of specificity in legume-Rhizobium associations. J. Appl. Bacteriol. 45, 165–194.

5 Broughton W J, Egeraat A W S M van and Lie T A 1980 Dynamics of *Rhizobium* competition for nodulation of *Pisum sativum* cv. Afghanistan. Can. J. Microbiol. 26, 562–565.

6 Chen A-P T and Phillips D A 1976 Attachment of Rhizobium to legume roots as the basis for specific interactions. Physiol. Plant. 38, 83–88.

7 Dazzo F B and Brill W J 1978 Regulation by fixed nitrogen of host-symbiont recognition in the Rhizobium-clover symbiosis. Plant Physiol. 62, 18–21.

8 Dazzo F B and Hubbell D H 1982 Control of root hair infection. *In* Nitrogen Fixation Vol. 2. Rhizobium. Ed. W J Broughton, Clarendon Press, Oxford, pp 274–310.

9 Dazzo F B and Hrabak E M 1982 Lack of a direct nitrate-trifolin. A interaction in the Rhizobium-clover symbiosis. Plant and Soil 69, 259–264.

10 Gibson A H 1980 Methods for legumes in glasshouses and controlled environment cabinets. *In* Methods for Evaluating Biological Nitrogen Fixation. Ed. F J Bergersen, John Wiley and Sons, Toronto, pp 139–184.

11 Kato G, Maruyana Y, Nakamura M 1980 Role of bacterial polysaccharides in the adsorption process of Rhizobium-pea symbiosis. Agric. Biol. Chem. 44, 2843–2855.
12 Munns D N 1968 Nodulation of *Medicago sativa* in solution culture. III. Effects of nitrate on root hairs and infection. Plant and Soil 29, 33–49.
13 Paau A S, Leps W T and Brill W J 1981 Agglutinin from alfalfa necessary for binding and nodulation by *Rhizobium meliloti.* Science 213, 1513–1515.
14 Steel R D G and Torrie J H 1960 Principles and Procedures of Statistics. McGraw-Hill, New York.
15 Vincent J M 1970 A Manual for the Practical Study of Root Nodule Bacteria. IBP Handb. No. 15, Blackwell Scientific Publications, Oxford and Edinburgh.