Regulation by Fixed Nitrogen of Host-Symbiont Recognition in the *Rhizobium*-Clover Symbiosis[1]

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ABSTRACT

Either NO_3^- (16 millimolar) or NH_4^+ (1 millimolar) completely inhibited infection and nodulation of white clover seedlings (*Trifoliin repens*) inoculated with *Rhizobium trifolii*. The binding of *R. trifolii* to root hairs and the immunologically detectable levels of the plant lectin, trifoliin, on the root hair surface had parallel declining slopes as the concentration of either NO_3^- or NH_4^+ was increased in the rooting medium. This supports the role of trifoliin in binding *R. trifolii* to clover root hairs. Agglutination of *R. trifolii* by trifoliin from seeds was not inhibited by these levels of NO_3^- or NH_4^+. The results suggest that these fixed N ions may play important roles in regulating an early recognition process in the *Rhizobium*-clover symbiosis, namely the accumulation of high numbers of infective *R. trifolii* cells on clover root hairs.

Nitrogen required by legumes can be satisfied by the symbiotic N_2-fixing activities of rhizobia in root nodules. Fixed N is one of the many environmental factors which can influence the development of this N_2-fixing symbiosis (10, 12, 22). The concentrations of NO_3^- and NH_4^+ which prevent nodulation are well below the concentrations necessary to cause injury to *Rhizobium* or the host plant (24). Studies using split root systems showed that inhibition of nodulation by NO_3^- is localized, affecting only those roots which have grown in the NO_3^- solution (24). Nitrate inhibits clover and alfalfa root hair deformation and infection by *Rhizobium* (15, 18, 23), indicating that fixed N effects occur long before onset of nodulation and symbiotic N_2 fixation. The legume host is the primary target for the fixed N effect (14, 18, 21). Nitrite reduced from NO_3^- by rhizobia plays no significant role in the process (13).

Recent studies have shown that a carbohydrate-binding protein, trifoliin, accumulates on root hairs of young clover seedlings (9). Trifoliin binds specifically to the microsymbiont of clover, *Rhizobium trifolii*, and may possibly serve as a symbiotic recognition protein for binding of *R. trifolii* to clover root hairs prior to infection (6–9). The purpose of this study was to examine the effects of fixed N on this recognition process occurring at the clover root hair surface.

MATERIALS AND METHODS

White clover seeds (*Trifoliin repens* var. Louisiana Nolin) were purchased from Johnson and Faris, Inc., Gainesville, Fla. Fluorescein isothiocyanate (FITC)[4]-conjugated γ-globulin of goat an-

tirabbit γ-globulin and fluorescent antibody mounting fluid (pH 9) were purchased from Difco. Purified agarose (type I) and BSA were from Sigma Chemical Co. All other reagents were analytical reagent grade available commercially. Solutions were prepared in glass-distilled H_2O. *R. trifolii* 0403 was obtained from P. S. Nutman, and was grown on plates for 5 days at 30 C with 1% (w/v) mannitol as the carbon source (3). Stainless steel wire mesh (size CX-16) was purchased from Small Parts, Inc., Miami, Fla. Trifoliin from seeds and rabbit antitrifoliin antibody were prepared as previously described (9). Cytofluorimetric measurements of root surface trifoliin were made with a Zeiss fluorescence microscope (HBO-200 lamp, KG1 BG 38, BG 12, and No. 50 barrier) using epiillumination and coupled to a model 520 P photometer (Photovolt Corp., New York).

Infection and Nodulation Studies. Seeds were surface-sterilized (19), spread on sterile water-agar plates (1.2% [w/v]purified agar), and germinated into humid air. Twenty-four-hr-old seedlings were inoculated with 5-day-old *R. trifolii* (2×10^7 viable cells) in Fahraeus solution (0.2 ml) supplemented with KNO_3 or NH_4^+ acetate on slide assemblies (11) and on sterile slants containing 1% (w/v) agarose. The concentration of NO_3^- or NH_4^+ was the same in the inoculum and the reservoir. Seedlings on slide assemblies were examined for root hair infection and nodulation after 6 days, while seedlings on agarose slants were examined for nodulation after 22 days. Slide assemblies and agarose slant cultures were replicated with four and six seedlings, respectively, for each concentration of NO_3^- or NH_4^+.

Root Growth Conditions. Roots were grown in a sterile chamber (17). Surface-sterilized seeds were embedded in sterile 0.7% (w/v) agarose. Blocks ($15 \times 15 \times 3$ mm) containing 10 embedded seeds were transferred to autoclaved stainless steel supports positioned just above 20 ml of Fahraeus seedling medium (11) in glass tubes (38×100 mm) covered with 50-ml glass beakers. The rooting medium was supplemented prior to planting with filter-sterilized NH_4^+ (as NH_4^+ acetate) and NO_3^- as (KNO_3) with each concentration in duplicate chambers. The chambers were incubated in a plant growth room at 23 to 25 C, with an 18-hr photoperiod, and a light intensity of 300 $\mu E/m^2$ sec. After 4 days, the primary root radicles had grown through the wire mesh into the rooting medium and to a length of approximately 15 mm. The agarose prevented the seed coats from falling into the rooting medium. Care was taken to avoid disturbing the seedlings during growth.

Bacterial Adsorption Assays. After 4 days of seedling growth, the nutrient medium was removed by syringe and needle. The seedlings were transferred on the wire mesh to tubes containing 10-ml suspensions of *R. trifolii*. The cells had been washed twice by centrifugation at 27,000g and resuspended at 10^{10} cells/ml in M buffer (0.2 mM K_2HPO_4-KH_2PO_4, 140 mM NaCl, 1 mM $MgSO_4$, 0.15 mM $MnCl_2$, 0.5 mM $CaCl_2$, pH 7.2). The roots were immersed in the bacterial suspensions, the tubes were covered with 50-ml beakers and lightly shaken at 30 C for 1 hr. The wire supports were then removed from the tubes. The roots were excised below the wire mesh, rinsed gently with filter-sterilized Fahraeus solu-

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[4] Abbreviation: FITC:fluorescein isothiocyanate.

tion, and examined microscopically under dark field illumination. Root adsorption assays used 5-day-old washed *R. trifolii* (5 × 10⁶ cells/24 hr seedling) in modified Fahraeus assemblies (8) supplemented with NO_3^- or NH_4^+ in the inoculum and the reservoir.

Measurement of Trifoliin on Seedling Roots. Four-day-old roots were cut below the wire mesh, rinsed with filter-sterilized Fahraeus solution, and incubated with 1 ml of rabbit antitrifoliin antiserum (heat-inactivated at 56 C for 30 min to destroy complement activity and diluted 1:20 with M buffer) in plastic tubes (12 × 75 mm, Falcon, Oxnard, Calif.) with intermittent shaking at 37 C. After 1 hr, the antiserum was removed, the roots were rinsed three times with M buffer, and then incubated with FITC-labeled goat antirabbit γ-globulin (0.4 ml, diluted 1:5 in M buffer). After 1-hr incubation at 37 C, the roots were washed three times with filtered Fahraeus solution, mounted on glycerol, and examined for maximum immunofluorescence in the root hair region (9). Immunofluorescent light intensity, measured as photovolts/mm², was corrected by subtracting background autofluorescence (15–20 v/mm²) and nonspecific binding of FITC-labeled goat antirabbit γ-globulin following preincubation of roots with normal, preimmune rabbit serum. Previous studies (9) showed that the nonspecific binding of goat antirabbit γ-globulin following preimmune serum corresponded to 0.7% of the relative maximal value of the homologous immune reaction on the roots, illustrating the high degree of specificity of the indirect immunofluorescence reaction.

Trifoliin Quantitative Agglutination Assay. Trifoliin was incubated with M buffer containing 16 mM KNO_3 or 1 mM NH_4^+ acetate for 1 hr at 30 C, and then assayed for agglutination of *R. trifolii* using M buffer (6, 9) containing NO_3^- or NH_4^+ as the diluent. Controls for cell autoagglutination in the modified M buffer were included. Protein was measured according to Lowry (16) using BSA as the standard.

RESULTS

Concentration of fixed N that Inhibits Infection and Nodulation. The lowest concentrations of either NH_4^+ acetate or KNO_3 which completely inhibited clover root hair infection and nodulation by *R. trifolii* were 1 and 16 mM, respectively. Ljunggren (15) found that similar concentrations of NO_3^- completely inhibited infection of white clover by *R. trifolii*. The rooting solutions were buffered to pH 6 ± 0.2. In the absence of fixed N, inoculated seedlings had an average of 12 infection threads and 2 nodules by 6 days, and 7 nodules by 22 days. No infection threads or nodules formed on uninoculated plants. Stunted seedling growth was not observed with seedlings grown in 20 mM NO_3^- or below 10 mM NH_4^+.

Adsorption of *R. trifolii* to Clover Root Hairs in the Presence of Fixed N. High numbers of *R. trifolii* accumulated (6, 9) at the tips of clover root hairs following 1-hr incubation in N-free medium with a dense inoculum (10¹⁰ cells/ml). Similar accumulation has been noted with other legume systems (4, 23). *R. trifolii* did not accumulate within 1 hr on seedling root hairs of plants grown in nutrient solutions containing fixed N (Fig. 1, A and B).
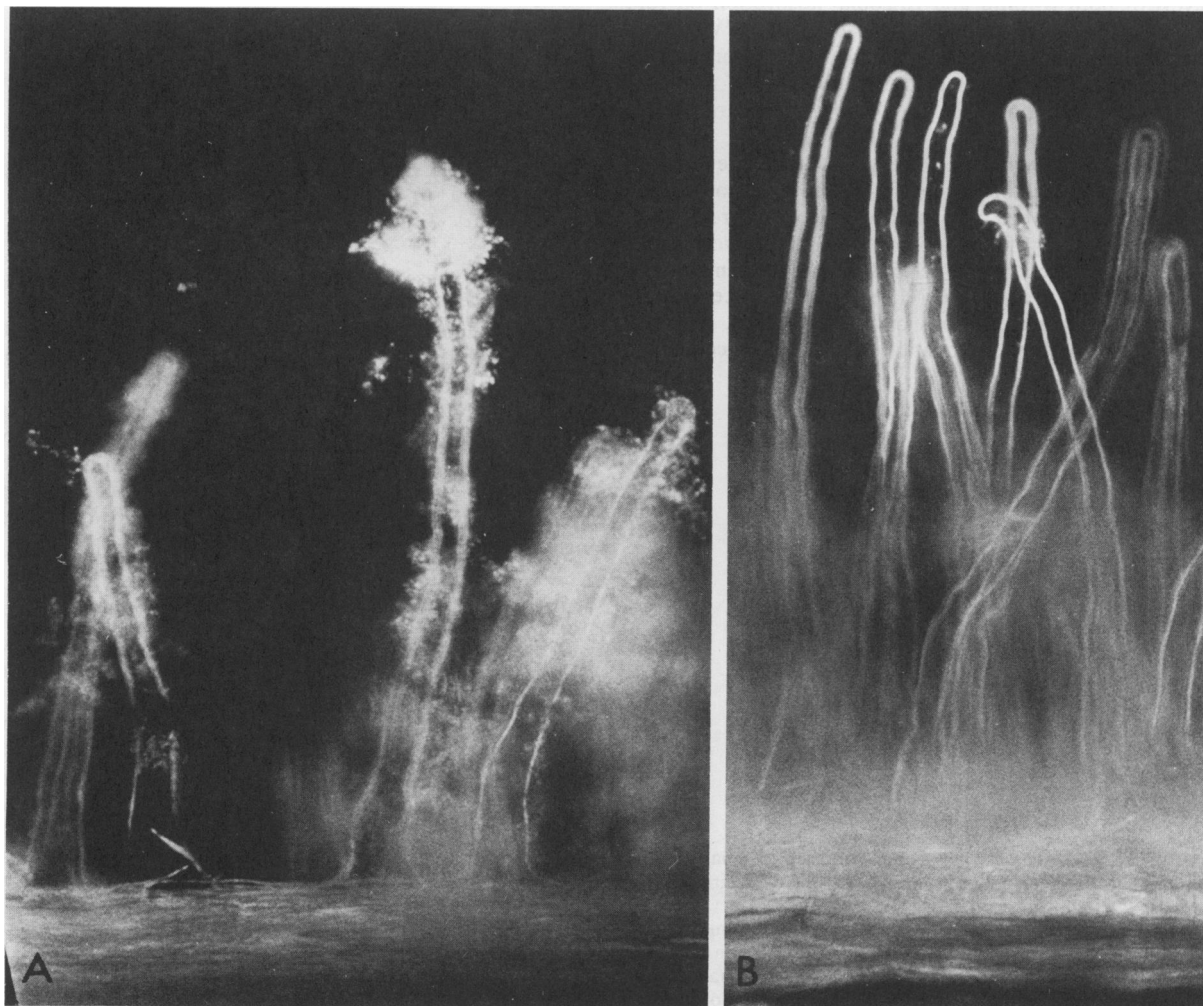

FIG. 1. Adsorption of *R. trifolii* to clover root hairs. Roots were grown in N-free Fahraeus solution (a) or Fahraeus solution supplemented with 16 mM KNO_3 (b), and then incubated with a washed, heavy inoculum for 1 hr. Roots were rinsed with Fahraeus solution and examined under dark field illumination.

These observations prompted us to look in more detail at the effect of fixed N on the quantitative adsorption of R. trifolii to clover root hairs. We used an assay (8) which employs a relatively low inoculum (5 × 10⁶ cells/plant) and only scores those bacterial cells with their surfaces in physical contact with the root hair cell wall. Microscopic counts of bacteria on root hairs 200 µm in length allowed comparisons based on standard surface areas of root hairs in similar states of physiological development (8). The results are shown in Figures 2 and 3. In the absence of exogenous fixed N, approximately 24 cells were adsorbed/root hair 200 µm in length. The adsorption of cells to root hairs was reduced to two to four cells at concentrations of NO_3^- or NH_4^+ that completely inhibit infection. This is the same number of cells that binds to root hairs within 12 hr if noninfective R. trifolii mutant strains, heterologous rhizobia which do not infect clover, or heat-killed infective R. trifolii are used (8). These low counts are subtracted for correction since they are due to nonspecific attachment to root hairs.

Measurement of Trifoliin on Roots. Since fixed N, like 2-deoxyglucose (8), inhibits rhizobial adsorption to clover root hairs, we wondered if either NH_4^+ or NO_3^- affected the distribution of trifoliin on clover seedling roots. Relative levels of immunologically detectable trifoliin were measured on the root hair region of clover seedlings grown in nutrient solutions containing fixed N. The results are shown in Figures 2 and 3. The relative levels of trifoliin and of R. trifolii adsorption to root hairs had parallel declining slopes as the concentrations of fixed N were increased in the rooting medium. It is interesting to note the small rise in relative levels of both trifoliin and rhizobial adsorption on root hairs at the intermediate concentration of 1 mM NO_3^-. We do not know to what extent this stimulation of root hair adsorption by low NO_3^- concentrations relates to the stimulation of root hair infection (5) and nodulation (20, 25) by low concentrations of NO_3^-.

Controls to Test for Counter-ion Effects. To test if the decrease in immunologically detectable levels of trifoliin or adsorption of R. trifolii to root hairs were due specifically to the presence of NO_3^- or NH_4^+, a number of counter-ion substitutions were made. The results are presented in Table I. The $NaNO_3$ and KNO_3 inhibited equally at 16 mM, as did $(NH_4)_2SO_4$, NH_4^+ acetate, and NH_4Cl at equimolar concentrations of NH_4^+ (1 mM). Negative counter-ion controls without effect at 16 mM were K^+ acetate and

FIG. 3. Effect of NH_4^+ on adsorption of R. trifolii to root hairs (———) and on immunologically detectable levels of trifoliin (---) in the root hair region of clover seedlings. Conditions as stated in Figure 2.

Table I. Effect of fixed N on root hair adsorption by R. trifolii on an immunologically detectable trifoliin on the root hair surface

Clover seedlings were grown in modified Fahraeus slide assemblies (8) and root chambers supplemented with the designated salts and then monitored for adsorption of R. trifolii to root hairs and immunofluorescent detection of trifoliin in the root hair region.

Salt supplement	Concentration (mM)	Root hair adsorption[a]	Relative immunofluorescence %
None	–	24	100
KNO₃	16	3	3
NaNO₃	16	3	3
NH₄⁺ acetate	1	3	3
NH₄Cl	1	4	4
(NH₄)₂SO₄	0.5	3	6
K⁺ acetate	16	25	103
Na⁺ acetate	16	26	102

[a] Cells per root hair 200 µm in length
[b] 980 photovolts per mm²

Na^+ acetate. These controls indicate that the effects observed were due to NH_4^+ and NO_3^-.

Effect of Fixed N on Agglutination of R. trifolii by Trifoliin. The specific agglutinating activity (1 × 10⁴ units/mg of protein) of purified trifoliin was the same regardless of the presence or absence of NO_3^- or NH_4^+, indicating that fixed N does not prevent agglutination of R. trifolii by trifoliin. Tubes in the dilution series containing the higher concentrations of trifoliin in M buffer plus 16 mM NO_3^- yielded stronger agglutination than corresponding concentrations of trifoliin in M buffer alone or M buffer containing 16 mM K^+ acetate.

DISCUSSION

The recognition process in the Rhizobium-clover symbiosis includes a preinfective event in which clover root hairs preferentially adsorb infective R. trifolii (8). This step occurs within 12 hr after rhizobial inoculation and may involve the specific binding of trifoliin on root hairs to unique carbohydrate structures found exclusively on the surface of R. trifolii (6–9). Specificity of Rhizobium-lectin interactions have been described with other symbiotic systems (1). The mechanism for specific adsorption of R. trifolii to clover root hairs is clearly distinguished from nonspecific mechanisms of rhizobial adsorption by its sensitivity to 2-deoxyglucose, and its restriction to infective R. trifolii cells (8). Immunochemical (7) and genetic (2) studies suggest that the receptors on R. trifolii and the clover root epidermis which bind trifoliin are similar. The results of this study indicate that fixed N ions in the rooting medium may play important roles in the regulation of this early recognition process. The inhibition by fixed N is expressed within hours after clover seed germination and it prevents R. trifolii from
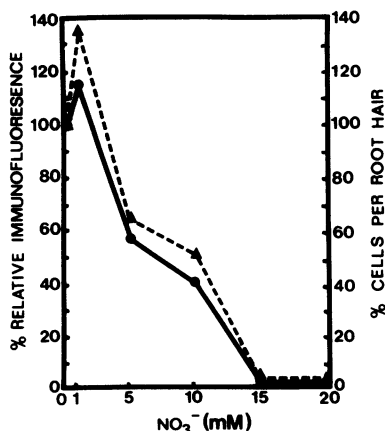

FIG. 2. Effect of NO_3^- on adsorption of R. trifolii to root hairs (———) and on immunologically detectable trifoliin (---) in the root hair region of clover seedlings. Bacterial adsorption was measured by direct counting and trifoliin was measured by cytofluorimetry using indirect immunofluorescence. Values from roots grown in N-free Fahraeus solution are taken as 100%, and represent 980 photovolts/mm² and 21 cells/root hair 200 µm in length. Points along the curve are means from 10 to 15 root hairs or seedling roots, standard deviations vary within 10% of the means. Values are corrected for nonspecific rhizobial adsorption, root autofluorescence, and nonspecific adsorption of FITC-labeled goat antirabbit γ-globulin.
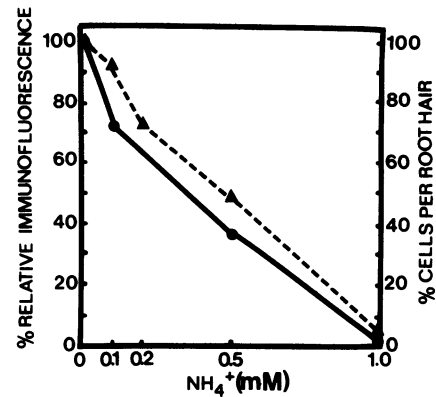
accumulating in high numbers on clover root hair surfaces. Since nodulation and N_2 fixation occur days to weeks after rhizobial adsorption to root hairs, their inhibition by fixed N are of secondary ecological consequences.

Exogenous fixed N supplied to the roots as NH_4^+ or NO_3^- regulates the level of immunologically detectable trifoliin on clover root hairs. The parallel curves of Figures 2 and 3 provide further support for the role of trifoliin in the specific adsorption of *R. triflii* onto clover root hair surfaces. Trifoliin can agglutinate *R. trifolii* in the presence of 16 mM NO_3^- or 1 mM NH_4^+, suggesting that these ions do not directly inactivate trifoliin. It is not yet known whether fixed N prevents accumulation or formation of trifoliin on the root hair surface or whether trifoliin that is present is somehow modified or masked.

Inhibition of N_2 fixation by fixed N in soil is viewed as an important limitation to the exploitation of biological N_2 fixation by cultivation of legumes in symbiosis with rhizobia. This study identifies one of the primary consequences of the presence of fixed N, and now allows us to study this fundamental process more directly at the molecular level.

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