Regulation by Fixed Nitrogen of Host-Symbiont Recognition in the Rhizobium-Clover Symbiosis

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ABSTRACT

Either NO3- (16 millimolar) or NH4+ (1 millimolar) completely inhibited infection and nodulation of white clover seedlings (Trifolium repens) inoculated with Rhizobium trifolii. The binding of R. trifolii to root hairs and the immunologically detectable levels of the plant lectin, trifolii, on the root hair surface had parallel declining slopes as the concentration of either NO3- or NH4+ was increased in the rooting medium. This supports the role of trifolii in binding R. trifolii to clover root hairs. Agglutination of R. trifolii by trifolii from seeds was not inhibited by these levels of NO3- or NH4+. 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 N2-fixing activities of rhizobia in root nodules. Fixed N is one of the many environmental factors which can influence the development of this N2-fixing symbiosis (10, 12, 22). The concentrations of NO3- and NH4+ 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 NO3- is localized, affecting only those roots which have grown in the NO3- 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 N2 fixation. The legume host is the primary target for the fixed N effect (14, 18, 21). Nitrite reduced from NO3- by rhizobia plays no significant role in the process (13).

Recent studies have shown that a carbohydrate-binding protein, trifolii, accumulates on root hairs of young clover seedlings (9). Trifolii 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 (Trifolium repens var. Louisiana Nolin) were purchased from Johnson and Faris, Inc., Gainesville, Fla. Fluorescein isothiocyanate (FITC)-4-conjugated γ-globulin of goat anti-
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 H2O. R. trifolii 0403 was obtained from P. S. Numan, 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. Trifolii from seeds and rabbit antitrifolii antibody were prepared as previously described (9). Cytocytometric measurements of root surface trifolii were made with a Zeiss fluorescence microscope (HBO-200 lamp, KG1 BG 38, BG 12, and No. 50 barrier) using epillumination 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 × 109 viable cells) in Fahraeus solution (0.2 ml) supplemented with KNO3 or NH4+ acetate on slide assemblies (11) and on sterile slants containing 1% (w/v) agarose. The concentration of NO3- or NH4+ 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 NO3- or NH4+.

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 × 15 × 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 NH4+ (as NH4+ acetate) and NO3- as (KNO3) 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 μE/m2 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 1010 cells/ml in M buffer (0.2 mM K2HPO4-KH2PO4, 140 mM NaCl, 1 mM MgSO4, 0.15 mM MnCl2, 0.5 mM CaCl2, 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 and placed in 50% (w/v) metaphosphoric acid.
tion, and examined microscopically under dark field illumination. Root adsorption assays used 5-day-old washed *R. trifolii* (5 x 10⁶ cells/24 hr seedling) in modified Fahraeus assemblies (8) supplemented with NO₃⁻ or NH₄⁺ 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 x 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 KNO₃ or 1 mM NH₄⁺ for 1 hr at 30°C, and then assayed for agglutination of *R. trifolii* using M buffer (6, 9) containing NO₃⁻ or NH₄⁺ 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 NH₄⁺ or KNO₃ 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 NO₃⁻ 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 NO₃⁻ or below 10 mM NH₄⁺.

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 (10⁶ 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 KNO₃ (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 x 10^6 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 Trifoliiin 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 trifoliiin on clover seedling roots. Relative levels of immunologically detectable trifoliiin 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 trifoliiin 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 trifoliiin 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 trifoliiin 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 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 Trifoliiin.** The specific agglutinating activity (1 x 10^6 units/mg of protein) of purified trifoliiin 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 trifoliiin. Tubes in the dilution series containing the higher concentrations of trifoliiin in M buffer plus 16 mM NO_3^- yielded stronger agglutination than corresponding concentrations of trifoliiin 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 trifoliiin 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 trifoliiin 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

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

<table>
<thead>
<tr>
<th>Salt supplement</th>
<th>Concentration (mM)</th>
<th>Root hair adsorption</th>
<th>Relative immunofluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>-</td>
<td>24</td>
<td>100</td>
</tr>
<tr>
<td>KNO_3</td>
<td>16</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>NaNO_3</td>
<td>16</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>(NH_4)_2SO_4</td>
<td>1</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>NH_4^+ acetate</td>
<td>1</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>NH_4Cl</td>
<td>1</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>(NH_4)_2SO_4</td>
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<td>3</td>
<td>3</td>
</tr>
<tr>
<td>K^+ acetate</td>
<td>16</td>
<td>25</td>
<td>100</td>
</tr>
<tr>
<td>Na^+ acetate</td>
<td>16</td>
<td>26</td>
<td>102</td>
</tr>
</tbody>
</table>

*cells per root hair 200 μm in length

*980 photosvolts per mm²
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 \(R. trifolii\) 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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