Anomalous nodulation of *Trifolium subterraneum* (subterranean clover) roots by *Rhizobium leguminosarum* 1020 was examined as a model of modified host-specificity in a *Rhizobium*-legume symbiosis. Consistent with previous reports, these nodules (i) appeared most often at sites of secondary root emergence, (ii) were ineffective in nitrogen fixation and (iii) were as numerous as nodules formed by an effective *Rhizobium trifolii* strain. *R. leguminosarum* 1020, grown on agar plates or in the clover root environment, did not bind the white clover lectin, trifoliin A. This strain did not attach in high numbers, and did not induce shepherd’s crooks or infection threads, in subterranean clover root hairs. However, *R. leguminosarum* 1020 did cause branching, moderate curling and other deformations of root hairs. The bacteria probably entered the clover root through breaks in the epidermis at sites of lateral root emergence. The anomalous nodulation was inhibited by nitrate. Only trace amounts of leghaemoglobin were detected in the nodules by Western blot analysis. The nodules were of the meristematic type and initially contained well-developed infection, bacteroid and senescent zones. Infection threads were readily found in the infection zone of the nodule. However, the bacteroid-containing tissue senesced more rapidly than in the effective symbiosis between subterranean clover and *R. trifolii* 0403. This anomalous nodulation of subterranean clover by *R. leguminosarum* 1020 suggests a naturally-occurring alternative route of infection that allows *Rhizobium* to enlarge its host range.

**INTRODUCTION**

*Rhizobium* is a genus of fast-growing Gram-negative bacteria which nodulate one or more species of legumes. In general, rhizobia which nodulate temperate legumes are highly specific in infection and nodulation of the roots of these plants. However, cross-infection and nodulation have been reported to occur among all the *Rhizobium* species (Graham, 1976), although these associations are usually ineffective (Vincent, 1980).

Several cases of cross-infection have been reported between the pea – *Rhizobium leguminosarum* and clover – *Rhizobium trifolii* cross-inoculation groups (Wilson, 1939; Kleczkowska *et al*., 1944; Schwinghamer, 1962; Graham & Parker, 1964; Hepper, 1978). For instance, a naturally occurring pea-nodulating strain, *R. leguminosarum* 1020, was found to form nodules on three species of clover (Hepper, 1978). The frequency of nodulation on *Trifolium subterraneum* (subterranean clover) was usually 100% and the ineffective nodules developed most often where a secondary root emerged from the primary root (Hepper & Lee, 1979). Hepper (1978) also reported that development of the symbiosis aborts at the point of bacterial release from the infection threads in nodules incited by *R. leguminosarum* 1020 on *Trifolium pratense* (red clover).

In this study, we investigated various steps in the anomalous nodulation process of
subterranean clover by *R. leguminosarum* 1020, from bacterial attachment and root hair responses to the ultrastructure of nodule development. The purpose of this work was to gain a more complete understanding of an anomalous *Rhizobium*—clover association so that the events required for a successful symbiosis can be better understood. A preliminary report of this work has been presented (Hrabak et al., 1984).

**METHODS**

*Cultivation of bacteria.* *R. trifolii* 0403 and *R. leguminosarum* 1020 were obtained, respectively, from P. Nutman and C. Hepper, Rothamsted Experimental Station, Harpenden, UK. *R. leguminosarum* 128C56 was obtained from J. Burton, Nitragin Co., Milwaukee, Wis., USA. *Escherichia coli* CR63 was obtained from L. Snyder, Michigan State University, East Lansing, Mich., USA. All bacteria were maintained at 30 °C on BIII defined agar medium (Dazzo, 1982). Single-cell suspensions were prepared by shaking the bacteria in HM-salts-Tween diluent (Cole State University, East Lansing, Mich., USA. All bacteria were maintained at 30 °C on BIII defined agar medium and C. Hepper, Rothamsted Experimental Station, Harpenden, UK. respectively.

Spontaneous rifampicin resistant (*Rif*) mutants of *R. leguminosarum* 1020 were isolated on BIII plates containing 20 μg rifampicin ml⁻¹. Plant inocula were prepared by growing bacteria on BIII plates for 5 d and then suspending the cells to a density of 1-2 x 10⁶ cells ml⁻¹ in nitrogen-free (N-free) Fahraeus mineral medium (Dazzo, 1982). For all experiments, pea and clover seedling roots were inoculated with 0.5 and 0.2 ml of the bacterial suspension, respectively.

*Cultivation of plants.* *T. subterranean* (subterranean clover) vars Woogenellup, Seaton Park, Clare, Geraldton, Yarlool, Northam, Mt Barker, Dalian, Esperance, Nangeela and Trikkala were obtained from M. George, University of Davis, Calif, USA. *Trifolium repens* (white clover) vars Louisiana Nolin and Ladino were obtained commercially. *Pisum sativum* (pea) vars 8617EP, Green Arrow, 9889F, 9888F, 44 per, ES2213, 5147DSP, 32 Alaska, 8615EP and 8221EP were obtained from D. Richins, Canners Seed Corp., Lewisville, Id., USA.

Clover seeds were surface-sterilized with acidified 0·1% (w/v) HgCl₂ and germinated in humid air at room temperature for 1-2 d. Seedlings were then aseptically transferred to enclosed sterile slants of N-free Fahraeus medium solidified with 0·8% (w/v) purified agar (Difco). In some cases, Fahraeus medium was supplemented with 25 mM-KNO₃ or KCl.

Pea seeds were surface-sterilized by treatment with 70% ethanol (v/v) for 1 min, followed by two 10 min washes with fresh commercial bleach (5·25% sodium hypochlorite) and 7-10 rinses with sterile water. The seeds were germinated in the dark at room temperature for 2 d on sterile, moistened filter paper in glass dishes. Seedlings (two per container) were transferred aseptically to enclosed glass tubes (4 x 30 cm) containing sterile vermiculite and N-free Fahraeus medium, or to sterile 125 ml Erlenmeyer flasks capped with foam plugs and containing 50 ml N-free Fahraeus medium solidified with 0·8% (w/v) purified agar. The flasks were covered with aluminium foil for 5 weeks, or until nodulation occurred, to avoid possible light inhibition of nodulation (Fred et al., 1932). As the shoots elongated, they were carefully withdrawn from the neck of the flask under sterile conditions and plugs were replaced. Sterile water or N-free Fahraeus medium was added to each flask culture as needed. All plants were incubated in a growth chamber with a 14 h photoperiod, a day/night temperature of 22 °C/20 °C and a light intensity of 26900 lx from mixed incandescent and fluorescent lamps.

*Pre-nodulation studies.* The ability of *R. leguminosarum* 1020 to bind the white clover lectin, trifoliin A, was examined by fluorescence microscopy (Dazzo & Hrabak, 1981). Bacteria were grown on BIII plates for 2, 3, 4, 5, 6, 7 or 12 d at 30 °C, harvested in 10 mM-potassium phosphate buffer (pH 7.2) containing 145 mM-NaCl and 1 mM-MgSO₄ (PBS), shaken gently and centrifuged at 2300 g for 20 min at 4 °C. Cells in the soft pellet were suspended in PBS and heat-fixed to fluorescent antibody slides (Clark Adams, Parsippany, NJ, USA). The bacteria were then treated sequentially with purified white clover lectin, trifoliin A, rabbit antiserum to trifoliin A, and fluorescein isothiocyanate-labelled goat anti-rabbit gamma globulin (Miles Labs, Elkhart, Ind., USA) diluted 1:5 in PBS (Dazzo & Hrabak, 1981).

The *in situ* binding of trifoliin A to free bacteria, incubated for 6 d in the root environment of Louisiana Nolin white clover in Fahraeus slide cultures without agar, was examined by indirect immunofluorescence microscopy using rabbit anti-trifoliin A antiserum as the primary antibody (Dazzo et al., 1982). Attachment of bacteria to roots of intact clover seedlings was examined qualitatively by indirect immunofluorescence microscopy and quantitatively by phase-contrast microscopy. *R. leguminosarum* 1020 or *R. trifolii* 0403 were inoculated on Clare subterranean clover seedlings on N-free Fahraeus agar slants and on Louisiana Nolin white clover seedlings in Fahraeus slide cultures. For fluorescence microscopy, seedlings were removed 17 h after inoculation and rinsed by gently vortexing with 5 ml sterile Fahraeus medium. The bacteria attached to the seedling roots were located by immunofluorescence microscopy using homologous rabbit anti-*Rhizobium* antiserum (1 mg ml⁻¹ in Fahraeus medium) (Dazzo et al., 1984). For quantitative attachment studies, roots were rinsed in Fahraeus medium as described above, cut into 5-8 mm segments and, when necessary, cut
Anomalous nodulation of subterranean clover

longitudinally. They were then mounted in Fahraeus medium and examined by phase-contrast microscopy. The bacteria firmly attached to root hairs about 200 µm in length were counted (Dazzo, 1982). The samples were coded so that the observer who examined the roots did not know which bacterial strain was used.

Deformation of root hairs was examined by phase-contrast microscopy along the optical median plane of each root on Louisiana Nolin white clover and Clare subterranean clover seedlings which had been incubated with bacteria on Fahraeus agar slants for 2, 5, 7, 9 and 12 d (Dazzo, 1982).

Root hairs of Clare subterranean clover were examined for infection thread formation after incubation on Fahraeus agar slants for 4, 8, 14, 16 or 24 d with *R. leguminosarum* 1020 or for 1, 2, 3, 4, 5 or 8 d with *R. trifolii* 0403. Roots were processed in three different ways in an attempt to detect infected root hairs. For phase-contrast microscopy, roots were cut into segments as described above, rinsed on the slide and mounted in Fahraeus medium. For bright-field microscopy, roots were stained for 30 min with 0.01 % (w/v) methylene blue in Fahraeus medium (Vasse & Truchet, 1984) and rinsed exhaustively with Fahraeus medium before mounting for observation. For epifluorescence microscopy, seedlings were first cleared in 10 mM-Tris/HCl (pH 7-5) containing 6 M-urea, 100 mM-NaCl and 1 mM-EDTA. Cleared roots were excised, rinsed in water, stained for 30 s with filter-sterilized acridine orange (30 µg ml⁻¹) in 100 mM-sodium phosphate buffer (pH 8-0), rinsed in buffer, mounted in buffer containing 145 mM-NaCl, and examined using fluorescent isothiocyanate-epifluorescence optics (Bohlool, 1985).

Post-nodulation studies. Roots of clover and pea plants grown under microbiologically controlled conditions in agar slants and flask cultures, respectively, were examined three times per week and the date of emergence of the first nodule was recorded. At the end of the incubation period, the plants were removed from the agar. The total number of nodules per plant and the position of the nodules, whether on primary or secondary roots or at the point of secondary root emergence, were noted. For plant dry weight determinations, the roots were gently rinsed in water to remove agar and the whole plant was dried in a pre-weighed aluminium weigh dish at 70 ºC for at least 18 h. The dishes were cooled to room temperature in a desiccator, reweighed and the plant dry weight determined by difference. The nitrogen-fixing activity of nodulated roots was estimated by the acetylene reduction technique. Roots were removed from the agar, separated from the shoot and incubated for 1-3 h in stopped serum vials containing 1 atm of humid air with 0-1 atm acetylene. Ethylene was detected by flame-ionization gas chromatography on a Porapak N column (Waters Associates, Milford, Mass., USA) at 70 ºC.

Pea or clover root nodules were surface sterilized with 0.1 % HgCl₂ and bacteria were isolated by plating nodule squashes on BIII medium.

For leghaemoglobin detection by SDS-PAGE, subterranean clover root nodules were harvested at various times after inoculation and were homogenized in a mortar at 4 ºC in 25 mM-Tris/HCl (pH 7-5) containing 0-3 M-sorbitol and 0-1 g insoluble poly-vinyl-pyrrolidone per g nodules. Cell debris and bacteroids were removed by filtering the homogenate through Miracloth (Calbiochem-Behring) followed by centrifugation for 10 min at 9200 g. Soluble proteins were salted out of the supernatant with ammonium sulphate at 50, 80 and 100 % saturation (Green & Hughes, 1955) and pelleted by centrifugation at 13200 g for 10 min at 4 ºC. The pellets were redissolved in PBS and dialysed against PBS overnight at 4 ºC. SDS-PAGE was done according to Laemmli (1970) in slab gels containing 12 % acrylamide; the gels were silver-stained by the method of Morrissey (1981), modified by eliminating the glutaraldehyde and diithiothreitol steps.

For leghaemoglobin detection by Western blotting, root nodules were harvested 40 d after inoculation and homogenized in a mortar with 1 ml 50 mM-Tris/HCl (pH 7-5) containing 0.1 g insoluble polyvinylpyrrolidone per g nodule wet weight. The homogenate was filtered through Miracloth and centrifuged for 10 min at 10000 g. The supernatant was prepared in a similar way using 4-d-old seedlings germinated in Petri dishes. Protein concentrations were measured using the Bio-Rad protein assay dye reagent concentrate with bovine serum albumin as standard. After electrophoresis in 15 % SDS-polyacrylamide gels, the proteins were electrophoretically transferred to a nitrocellulose membrane (BA85, Schleicher and Schuell) using a Bio-Rad Trans Blot cell, with 20 mM-Tris containing 150 mM-glycine and 20 % (v/v) ethanol (pH 8-3) as the transfer buffer. The nitrocellulose membranes were incubated with antiserum raised against purified pea leghaemoglobin components (Bisseling et al., 1979). Protein A from *Staphylococcus aureus*, labelled with 125 I, was bound to the immune complexes on the membranes and visualized by autoradiography (Bisseling et al., 1983).

For microscopic examinations, excised nodules were fixed in 4 % (v/v) glutaraldehyde in 0-2 M-sodium cacodylate buffer (pH 7-2), post-fixed in 1 % (w/v) osmium tetroxide, dehydrated in ethanol, embedded in Epon 812, and thin-sectioned on an Ultratome III (LKB, Sweden). Sections (2-3 µm) were mounted on glass slides, stained with basic fuchsin-methylene blue (Huber et al., 1968) and examined by bright-field photomicrography using a green filter to enhance contrast. Ultrathin sections were post-stained with lead citrate/uranyl acetate and examined with a Philips 300 transmission electron microscope.

Statistics. All statistical differences were measured at the 95 % confidence level using a Student’s t test, with t calculated as the difference in the two means divided by the standard error of the difference of the two means.
RESULTS

Preliminary screening of pea and subterranean clover varieties

All 11 varieties of subterranean clover formed nodules within 4 weeks after inoculation with *R. leguminosarum* 1020. Based on seed viability, number of nodules and plant appearance under our growth conditions, four varieties (Clare, Woogenellup, Seaton Park and Geraldton) were selected for further study. A preliminary screen of the 10 pea varieties inoculated with *R. leguminosarum* 1020 was done using vermiculite tube culture. When examined 7 weeks after inoculation, seven of the 10 pea varieties had formed nodules. Based on plant vigour, internode length and number of nodules formed, variety 9888F was selected for all subsequent studies.

Culture purity

Single, isolated colonies of *R. leguminosarum* 1020, obtained by streaking from cultures grown on agar medium or by plating single-cell suspensions dispersed in HM-salts-Tween diluent, displayed a uniform morphology on BIII plates. Spontaneous Rif<sup>r</sup> mutants of *R. leguminosarum* 1020 also had uniform colony morphology when streaked on BIII agar.

Each of the *R. leguminosarum* 1020 nodule isolates from pea and from the four varieties of subterranean clover was able to nodulate peas and all four varieties of subterranean clover. Bacteria reisolated from these nodules were still able to nodulate both peas and subterranean clover. Pea and subterranean clover plants were also nodulated by the Rif<sup>r</sup> mutants of *R. leguminosarum* 1020, and bacteria isolated from these nodules were Rif<sup>r</sup>.

To ensure that surface-sterilization of nodules was complete, some excised nodules were incubated for 15 min with a suspension of *E. coli*, then surface sterilized, and rolled across BIII plates. No colonies of *E. coli* were detected after incubation of the plates at 30 °C for 5 d.

Confirmation of nodulation parameters of *R. leguminosarum* 1020

The nodulation characteristics of pea and clover plants inoculated with *R. leguminosarum* 1020, *R. leguminosarum* 128C56 and *R. trifolii* 0403 were compared (Table 1). Peas nodulated effectively with *R. leguminosarum* 1020 and *R. leguminosarum* 128C56 but never nodulated with *R. trifolii* 0403. Pea nodules generally developed on secondary roots and few were found at sites of lateral root emergence. Dry weights of peas nodulated by *R. leguminosarum* 1020 were not significantly different from peas nodulated by the highly effective *R. leguminosarum* strain 128C56 used as a positive control, but both were significantly higher than those of uninoculated plants. Acetylene reduction assays done throughout the growing period showed that all of the subterranean clovers were nodulated effectively with *R. trifolii* 0403 and ineffectively with *R. leguminosarum* 1020, although the dry weights of these plants did not differ significantly. Subterranean clover was not nodulated with *R. leguminosarum* 128C56. In the subterranean clover – *R. leguminosarum* 1020 association, nodules took significantly longer to appear and developed more frequently at sites of lateral root emergence. However, the average number of nodules produced on clover by *R. leguminosarum* 1020 and *R. trifolii* 0403 by 50 d after inoculation was not significantly different. No nodules formed on uninoculated pea or clover plants.

Pre-nodulation plant–bacteria interactions

*R. leguminosarum* 1020, grown on agar plates for 2–12 d or in the white clover root environment of Fahraeus slide cultures for 6 d, did not bind detectable levels of the white clover lectin, trifoliin A, as measured by the immunofluorescence assay.

Epifluorescence microscopy showed that both *R. trifolii* 0403 and *R. leguminosarum* 1020 attached to undifferentiated epidermal cells in the root hair region of subterranean clover seedlings within 17 h after inoculation, but that, in contrast, attachment to root hairs was considerably greater for *R. trifolii* 0403 (Fig. 1a, b). This difference in attachment was confirmed by quantitative microscopic counts of both strains on root hairs which were about 200 μm long. *R. trifolii* 0403 attached to white clover root hairs at 25·0 ± 0·7 cells per root hair and to subterranean clover at 25·6 ± 0·6 cells per root hair. In contrast, *R. leguminosarum* 1020
detected by epifluorescence microscopy 17 h after inoculation on 2-d-old seedlings. (a) *R. trifolii* 0403 cells attached to root hairs and undifferentiated epidermal cells. (b) *R. leguminosarum* 1020 cells attached to undifferentiated root epidermal cells. Bars, 100 μm.

attached to white clover root hairs at 3.1 ± 0.4 cells per root hair and to subterranean clover at 5.8 ± 0.3 cells per root hair.

Root hairs of both Ladino white clover and Clare subterranean clover seedlings developed marked curling (shepherd’s crooks) as early as 2 d after inoculation with *R. trifolii* 0403. Typical root hair deformations in response to *R. trifolii* 0403 included moderate curling, branching and marked curling (Fig. 2a, b). In contrast, shepherd’s crooks did not consistently appear on either clover host inoculated with *R. leguminosarum* 1020, even by 12 d after inoculation. However, *R. leguminosarum* 1020 did induce other deformations, including moderate curling and branching of root hairs (Fig. 2c, d). Root hairs of uninoculated clover plants were straight (Fig. 2e, f) and showed no deformations like those of inoculated plants.

**Examination of root hairs for infection threads**

Three microscopic methods were used to examine root hairs of Clare subterranean clover for infection thread formation as a mechanism of infection by *R. leguminosarum* 1020. Under our growth conditions, no infection threads were detected by these methods in any root hairs on either primary or secondary roots or in root axils of 38 plants after incubation with the bacteria for 4, 8, 14, 16 or 24 d, despite the fact that nodules formed on these plants during this time period. Secondary roots of subterranean clover were smaller in diameter and produced a lower density of root hairs than primary roots, thus facilitating the search for infection threads in these root hairs. By 14 d after inoculation, swellings could be detected on secondary roots (Fig. 3). It was possible to inspect each root hair on the surface of and in the vicinity of nine such swellings which, when cleared and stained with acridine orange, could be recognized as pre-emergent nodules and not lateral roots. Even in these areas where root nodules were beginning to develop, infection threads were not found within any of the root hairs examined. In contrast to these negative results with *R. leguminosarum* 1020, all three methods readily revealed infection threads within root hairs on primary and secondary roots of Clare subterranean clover inoculated with the *R. trifolii* 0403 control. Infected root hairs on the primary root were first detected after 3 d incubation with *R. trifolii* 0403 and an average of nine infections per plant were seen after 5 d.

**Effect of nitrate on anomalous nodulation**

Seedlings of subterranean clover (vars Clare and Woogenellup) grown on agar slants of Fahraeus medium supplemented with 25 mM-KNO₃ did not nodulate by 7 weeks after inoculation with *R. leguminosarum* 1020 or *R. trifolii* 0403. Nodulation by these strains occurred normally in the presence of 25 mM-KCl.
Anomalous nodulation of subterranean clover

Fig. 3. Swelling representing a nodule primordium on a secondary root of Clare subterranean clover incubated for 16 d with R. leguminosarum 1020. Bar, 100 µm.

Fig. 4. Autoradiogram of a Western blot incubated with antiserum raised against purified pea leghaemoglobins and 125I-labelled protein A to detect immune complexes. Soluble cytoplasmic proteins were isolated from pea nodules containing R. leguminosarum 1020 (lane a), uninoculated pea roots (lane b), Clare subterranean clover nodules containing R. leguminosarum 1020 (lane c), uninoculated Clare subterranean clover roots (lane d), Clare subterranean clover nodules containing R. trifolii 0403 (lane e), uninoculated Louisiana Nolin white clover roots (lane f) and Louisiana Nolin white clover nodules containing R. trifolii 0403 (lane g). The autoradiographic exposure of lane c was approximately five times longer than that of the other lanes. Molecular weight markers included [14C]methylated bovine serum albumin (69000), ovalbumin (46000), carbonic anhydrase (30000) and lysozyme (14300).

Leghaemoglobin analyses

Since nodules on subterranean clover formed by R. leguminosarum 1020 were white and did not reduce acetylene at 30–90 d after inoculation (Table 1), soluble plant cytoplasmic nodule proteins were separated by SDS-PAGE to try to detect leghaemoglobin. Although leghaemoglobin was found to be a major component of subterranean clover nodule extracts from R. trifolii 0403, a corresponding leghaemoglobin band was not seen in the nodule extracts from R. leguminosarum 1020, even when the nodule proteins had been concentrated by ammonium sulphate precipitation and the gel was silver-stained. A Western blot confirmed that leghaemoglobin was readily detected in nodules from the homologous symbioses between R. leguminosarum 1020 and pea (Fig. 4, lane a), or between R. trifolii 0403 and clover (Fig. 4, lanes e and g). A five times longer autoradiographic exposure of this blot detected traces of leghaemoglobin (about molecular wt 14000) in subterranean clover nodules formed by R. leguminosarum 1020 (Fig. 4, lane c). Thus, traces of leghaemoglobin were found in subterranean clover nodules formed by R. leguminosarum 1020 by Western blot analysis but not by SDS-PAGE. Proteins isolated from uninfected root tissue did not react with the anti-leghaemoglobin serum in Western blot analysis (Fig. 4, lanes b, d and f).

Fig. 2. Deformation responses of root hairs of Ladino white clover (a, c, e) and Clare subterranean clover (b, d, f) plants to inoculation with either R. trifolii 0403 or R. leguminosarum 1020. (a, b) Root hairs 5 d after inoculation with R. trifolii 0403. Note marked curling (shepherd’s crooks) (arrow and inset) as well as moderate curling, undulation and branching of root hairs. (c, d) Root hairs 5 d after inoculation with R. leguminosarum 1020. Note the branching and undulation, but only moderate curling of root hairs. (e, f) Root hairs on uninoculated 5-d-old plants. Bars, 100 µm (except inset of (a); bar, 20 µm).
Table 1. Nodulation characteristics of *R.* *leguminosarum* 1020, *R.* *leguminosarum* 128C56 and *R.* *trifolii* 0403 on peas and subterranean clover

*R.* *trifolii* 0403 did not nodulate peas and *R.* *leguminosarum* 128C56 did not nodulate subterranean clover. Data are expressed as mean ± SEM. Values in parentheses represent the number of plants in the samples. Two results followed by the same letter are significantly different at the 95% confidence level.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Bacterial inoculum</th>
<th>Days to first nodule</th>
<th>Mean no. of nodules per plant</th>
<th>Percentage of nodules at secondary root emergence</th>
<th>Nitrogenase activity*</th>
<th>Plant dry weight (mg)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pea</td>
<td><em>R.</em> <em>leguminosarum</em> 1020</td>
<td>24 ± 1.2 (25)</td>
<td>23 ± 2.4 (29)</td>
<td>13 ± 2.2 (31)</td>
<td>547 ± 97 (35)</td>
<td>247 ± 24.0 (17)</td>
</tr>
<tr>
<td>Pea</td>
<td><em>R.</em> <em>leguminosarum</em> 128C56</td>
<td>22 ± 0.9 (21)</td>
<td>15 ± 3.1 (28)</td>
<td>5 ± 1.5 (29)</td>
<td>746 ± 157 (32)</td>
<td>246 ± 26.8 (14)</td>
</tr>
<tr>
<td>Clover‡</td>
<td><em>R.</em> <em>leguminosarum</em> 1020</td>
<td>21 ± 0.7 (111)</td>
<td>6 ± 0.3 (86)</td>
<td>84 ± 2.4 (83)</td>
<td>0 (135)</td>
<td>14 ± 0.7 (20)</td>
</tr>
<tr>
<td>Clover‡</td>
<td><em>R.</em> <em>trifolii</em> 0403</td>
<td>15 ± 0.7 (59)</td>
<td>5 ± 0.4 (52)</td>
<td>11 ± 2.6 (48)</td>
<td>5 ± 0.8 (12)</td>
<td>13 ± 0.7 (24)</td>
</tr>
</tbody>
</table>

* Acetylene reduction values are expressed as nanomoles ethylene produced per plant per h. Peas were assayed about 40 d after inoculation and clover inoculated with *R.* *trifolii* 0403 at about 50 d. Clover inoculated with *R.* *leguminosarum* 1020 were assayed at various times from 30–90 d after inoculation.

† Plants were weighed about 40 d after inoculation for peas and 50 d for clover. The dry weight of non-nodulated peas and clover plants was 154 and 15 mg, respectively.

‡ *Trifolium subterraneum* vars Clare and Woogenellup.
Microscopy of nodules

Nodules formed by R. trifolii 0403 and R. leguminosarum 1020 on subterranean clover plants were compared by light and electron microscopy. Both bacteria formed typical meristematic-type nodules (Fig. 5), characterized by an apical meristem in zone I, an infection zone II in which bacteria-containing infection threads penetrated plant cells and the bacteria were released, a bacteroid zone III where bacteria were transformed into enlarged, pleomorphic bacteroids, and finally, a senescent zone IV where bacteroids and plant cells had degenerated.

Electron microscopic examination of nodules formed on subterranean clover by R. trifolii 0403 and harvested at 24, 30 and 40 d after inoculation had typical nodule development and bacteroid morphogenesis. In zone II, bacteria were released from infection threads in the plant cytoplasm and surrounded by a bilayer peribacteroid membrane (Fig. 6a). Enlarged and pleomorphic bacteroids, completely filling their peribacteroid membranes, developed in zone III (Fig. 6b). Proceeding from zone III to zone IV, the bacteroids were increasingly more vacuolated and their cytoplasm was more electron-dense (Fig. 6c), suggesting that they were degenerating. In the senescent zone IV, the plant cytoplasm lost contact with the cell wall and bacteroids were not observed (Fig. 6c).

At 24 d after inoculation, the ultrastructure of nodules formed by R. leguminosarum 1020 or R. trifolii 0403 on subterranean clover was not strikingly different. Bacterial release from infection threads, bacteroid formation, vacuolization of bacteroids and senescence of bacteroids and plant cells were all observed. The one difference was premature senescence of some R. leguminosarum 1020 bacteroids, recognized by their increased electron density and pleomorphism (Fig. 7a, b), at the proximal end of zone II and throughout zone III. In R. leguminosarum 1020 nodules harvested at 30 d after inoculation, premature senescence of some bacteroids was now evident in the distal part of zone II (Fig. 8), although the rest of the nodule and bacteroid morphology appeared similar to nodules harvested 30 d after inoculation with R. trifolii 0403.
Fig. 6. Transmission electron micrographs of Clare subterranean clover nodules 40 d after inoculation with *R. trifolii* 0403. (a) Bacteria released from the infection thread in zone II. Each cell is surrounded by a peribacteroid membrane (arrows). (b) Bacteroid zone III with many well-differentiated, pleomorphic bacteroids. (c) Degeneration of two plant cells in the senescent zone IV. On the left, the bacteroids contain many vacuoles and a dense cytoplasm. In the more senescent cell on the right, the plant cytoplasm is no longer in contact with the cell wall (arrows) and the cell contents are in disarray. Bars, 1 μm.

Fig. 7. Transmission electron micrographs of Clare subterranean clover nodules 24 d after inoculation with *R. leguminosarum* 1020. Note the increased electron density and loss of well-defined edges of the senescent cells. (a) Electron-dense, prematurely senescent bacteria (arrows) in the late (proximal) infection zone I1 (contrast with Fig. 6a). (b) Early senescence of some bacteria (arrows) in the bacteroid zone III (contrast with Fig. 6b). Bars, 1 μm.

(not shown). By 40 d after inoculation, many more senescent cells of *R. leguminosarum* 1020 were found in zone II and the cytoplasm of many other bacteria had an anomalous loss of electron density (Fig. 9a). Unlike *R. trifolii* 0403, two cells of *R. leguminosarum* 1020 were often found within the same peribacteroid membrane (contrast Fig. 6a with Fig. 9a). In zone III, another indication of premature senescence of *R. leguminosarum* 1020 bacteroids was their prominent vacuolization, which normally occurs only in the senescent zone IV with *R. trifolii* 0403 (contrast Fig. 6b and c with Fig. 9b). At 40 d after inoculation, the senescent zone IV almost completely filled some nodules. The only 'non-degenerating' bacterial cells were those still within the lumen.
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Fig. 8. Transmission electron micrograph of Clare subterranean clover nodules 30 d after inoculation with *R. leguminosarum* 1020, showing premature senescence of bacteria (arrows) in the early (distal) infection zone II. Infection threads (double arrows) are present in the nodule. Bar, 1 μm.

Fig. 9. Transmission electron micrographs of Clare subterranean clover nodules 40 d after inoculation with *R. leguminosarum* 1020. (a) Bacteria in the infection zone II showed the following signs of premature senescence: degeneration (single arrow), two bacteria in the same peribacteroid membrane (double arrows) and loss of electron density of the bacterial cytoplasm (triple arrows). (b) In the bacteroid zone III, all of the bacteroids show prominent vacuolization. (c) The interior of a highly senescent nodule where only bacteria within or very near an infection thread (double arrows) are still recognizable (arrows). Bars, 1 μm.

DISCUSSION

The development of a successful nitrogen-fixing symbiosis between *Rhizobium* and legumes involves a close interaction of bacterial and plant genetic traits. In this study, the ineffective and anomalous nodulation of subterranean clover by *R. leguminosarum* 1020 was investigated in depth to identify its similarities and differences with the normal and effective nodulation of infection threads or those very close to, and perhaps recently released from, the infection thread (Fig. 9c). Lysosome-like organelles were more abundant in nodules formed by *R. leguminosarum* 1020 than in nodules formed by *R. trifolii* 0403 (contrast Fig. 6a with Fig. 9a).
process by *R. trifolii* 0403, and to gain a better understanding of the events required for establishment of a successful symbiosis.

All evidence indicated that the culture of *R. leguminosarum* 1020 used in this report was pure. The *R. leguminosarum* 1020 culture displayed uniform colony morphology on BIII plates, even after repeated inoculation of plants with bacteria grown from single colonies and reisolation of the bacteria from pea and subterranean clover nodules. As a more stringent test, spontaneous Rif\(^+\) colonies of *R. leguminosarum* 1020, inoculated on both peas and subterranean clover, produced root nodules occupied by Rif\(^+\) cells. Bacteria isolated from these nodules were able to nodulate either peas or subterranean clover. This proves that the pea-nodulating *R. leguminosarum* strain can nodulate subterranean clover.

This paper confirms previous reports of anomalous nodulation of subterranean clover by *R. leguminosarum* 1020 (Hepper, 1978; Hepper & Lee, 1979) which showed that most nodules are formed at the junction of primary and secondary roots and that this ineffective symbiosis does not produce more nodules per plant than an effective *R. trifolii* strain. This latter result differs from the common observation that ineffective bacterial strains often form more nodules than effective strains (Dart, 1977). In the case of subterranean clover (Nutman, 1967), the variation in number of nodules per plant of any given variety was found to be small, but the mean number of nodules per plant differed among varieties and was inherited in a complex manner. Nodule number was also affected, usually independently, by the bacterial strain (Nutman, 1967). Therefore, the fact that the ineffective association between *R. leguminosarum* 1020 and either of the two varieties of subterranean clover roots inoculated with *R. leguminosarum* 1020 only after the emergence of lateral roots, and significantly later than when the inoculum was *R. trifolii* 0403, indicating a slower nodulation rate for *R. leguminosarum* 1020. The dry weights of subterranean clover plants inoculated with these two strains did not differ significantly when measured 40 d after inoculation, probably either because the cotyledons were still attached to the plants and could still provide combined nitrogen for plant development at this age or because *R. trifolii* 0403 is not as effective on subterranean clover as on white clover. Therefore, plant dry weights were not as useful a measure of ineffectiveness for subterranean clover as acetylene reduction under our growth conditions. Subterranean clover plants nodulated by *R. leguminosarum* 1020 were assayed periodically between 30 and 90 d after inoculation for nitrogen fixation but none was ever detected.

The significantly slower nodulation rate and higher frequency of nodule formation at lateral root emergence by *R. leguminosarum* 1020 prompted further investigation into the mechanism of infection and nodulation of subterranean clover. In general, the early stages of this anomalous infection process showed little interaction between the bacteria and the host plant. In contrast to *R. trifolii* 0403, *R. leguminosarum* 1020, grown on plates or in the clover root environment, did not bind to the white clover lectin, trifoliin A, or attach to white or subterranean clover root hairs above the background levels of 2–5 cells per 200 μm root hair, which are typical for certain non-infective *R. trifolii* strains or for heterologous rhizobia (Dazzo et al., 1976). It is not known whether subterranean clover produces a different root lectin which may be involved in the infection process with *R. leguminosarum* 1020. In previous studies (Dazzo et al., 1984), *R. leguminosarum* 1020 also did not display the 4–12 h sequence of phase 1A, 1B and 1C attachment to clover root hairs that is typical of *R. trifolii* 0403. In contrast, attachment of *R. leguminosarum* 1020 to undifferentiated root epidermal cells was similar to that seen with *R. trifolii* 0403, implying that receptors for trifoliin A are not necessary for attachment to this part of the epidermis. *R. leguminosarum* 1020 was able to cause branching, moderate curling and undulations of clover root hairs. Branching is considered to be only a moderately specific host response (Vincent, 1980). In general, *R. leguminosarum* strains induce more moderate curling and branching of clover root hairs than other heterologous rhizobia (Yao & Vincent, 1969; Dart, 1977) and this response is viewed as evidence of the close relation between these cross-inoculation groups. Neither marked curling (shepherd’s crooks) nor infection threads were found within any of the subterranean clover root hairs inoculated with *R. leguminosarum* 1020,
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even in the vicinity of pre-emergent and developing nodules, although infection threads were
present in emerged nodules. In contrast, infection threads were readily found in root hairs of
subterranean clover plants inoculated with *R. trifoli* 0403. These results suggest that two
different routes of infection can lead to nodule formation in subterranean clover. Frequent
formation of nodules at lateral root emergence without root hair infection suggests that *R.
leguminosarum* 1020 enters the root through breaks in the epidermis and then spreads throughout
the developing nodule in infection threads. This route of infection has also been demonstrated in
root nodule of *Aeschynomene americana* (Napoli et al., 1975), and is similar to the stem
nodulation of *Sesbania rostrata* (Tsein et al., 1983) which occurs when adventitious root
primordia break through the stem epidermis. However, the anomalous nodulation of subterranean
clover by *R. leguminosarum* 1020 differs from other modes of rhizobial infection with *Arachis hypogaea* (Allen & Allen, 1940; Chandler, 1978), *Stylosanthes* spp. (Ranga Rao, 1977; Chandler et al., 1982) and *Aeschynomene indica* (Arora, 1954) where infection threads have not
been found in the root hairs or nodules. Also, a *R. trifoli* strain which does not form infection
threads but produces ineffective nodules on both subterranean and white clover has been
reported recently (Plazinski & Rolfe, 1985). In addition, the infection process of subterranean
clover by *R. leguminosarum* 1020 differs from the nodulation of lima bean by *Rhizobium* sp.
127E15, where infection threads have been observed both in root hairs (Shantharam & Wong,
1982) and in undifferentiated epidermal cells (Bal & Wong, 1982). Since infection of
subterranean clover roots by *R. leguminosarum* 1020 does not occur through root hairs, it most
probably occurs at sites of epithelial damage or desquamation, or through undifferentiated
epidermal cells.

The nodulation of subterranean clover by *R. leguminosarum* 1020 and *R. trifoli* 0403 are
similar in that both are inhibited by nitrate (Dazzo & Brill, 1978). This is not surprising since all
of the recognized steps of root nodulation, with the exception of meristem formation, are
inhibited by combined nitrogen (Truchet & Dazzo, 1982).

Subterranean clover nodules formed by *R. leguminosarum* 1020 did not fix nitrogen and were
white, and no band corresponding to leghaemoglobin was apparent on SDS-PAGE gels.
However, low levels of leghaemoglobin could be detected using the more sensitive Western
blotting technique with anti-pea leghaemoglobin serum as the probe. Bisseling et al. (1983)
reported that ineffective pea nodules always contained small amounts of leghaemoglobin,
indicating that the presence of the rhizobia induced some leghaemoglobin synthesis. Govers et al.
(1985) found that leghaemoglobin mRNA is present in ineffective pea nodules at 30–60% of
the levels in effective nodules 13–20 d after inoculation. However, leghaemoglobin was only
detectable in the ineffective nodules around day 14, indicating that synthesis of leghaemoglobin
is post-transcriptionally controlled (Govers et al., 1985). Similar results have been reported with
ineffective soybean nodules (Verma et al., 1981). Leghaemoglobin is assumed to facilitate
diffusion of oxygen to the bacteroid (Appleby et al., 1975) and the concentration of
leghaemoglobin has often been correlated with levels of nitrogenase or with effectiveness
(Virtanen et al., 1947; Bergersen & Goodchild, 1973; Appleby, 1974; Bisseling et al., 1978).

Nodules produced by *R. leguminosarum* 1020 on red clover lacked well-defined zones and the
bacteria were not released from the infection threads (Hepper, 1978). However, nodules
produced by this strain on subterranean clover and examined ultrastructurally 24 d after
inoculation were quite similar to effective nodules induced by *R. trifoli* 0403. Nodules on
subterranean clover induced by both strains contained a zone of bacterial release from infection
threads, followed by a zone of bacteroid development. However, a unique feature of the
anomalous nodules was a rapidly advancing zone of senescence proceeding toward the
meristematic end of the nodule between 30 and 40 d after inoculation. Fusion of peribacteroid
membranes occurred and many lysosome-like organelles were present in the degenerating host
cells. The lysosomal system may be participating in the destruction of both the bacteria and
plant cell contents, as demonstrated previously for pea nodules (Truchet & Coulomb, 1973). In
comparison, these features are only found in the senescent zone of effective pea nodules (Truchet
& Coulomb, 1973) and of clover nodules formed by *R. trifoli* 0403.

Normal early development of nodules, followed by lack of persistence of the bacteroid state, is
a common feature of ineffective nodules from many legumes (Bergersen, 1955, 1957; Bergersen & Nutman, 1957; MacKenzie & Jordan, 1974; Bassett et al., 1977; Rolfe & Gresshoff, 1980; Vance & Johnson, 1983). Various explanations for this have been proposed, including nitrogen starvation of the plant (MacKenzie & Jordan, 1974) and failure of the bacteria to undergo the physiological changes necessary to maintain the bacteroid state (Bergersen, 1957). In the anomalous nodulation investigated here, bacteroids with apparently normal structure are formed but only traces of leghaemoglobin are produced. One interpretation could be that insufficient leghaemoglobin production causes a decreased oxygen flux into the nodule and the resulting oxygen limitation of the bacteroid tissue prevents nitrogen fixation and perhaps accelerates senescence. Lack of oxygen to Lupinus nodules greatly decreased their acetylene-reducing activity (Trinick et al., 1976).

A long-range goal of Rhizobium genetics is to broaden the host range of Rhizobium and develop new effective symbioses with other useful plants. This anomalous nodulation by R. leguminosarum 1020 represents a naturally occurring alternative route of infection of subterranean clover which is independent of root hair recognition and infection, but results in ineffective nodules. Recently, we have also observed that R. leguminosarum 1020 readily forms ineffective nodules on bean (Phaseolus vulgaris) (E. M. Hrabak & J. Maya-Flores, unpublished observation). Perhaps this strain can be useful to identify genes allowing Rhizobium to breach the barrier of host specificity in the nitrogen-fixing Rhizobium-legume symbiosis.

We thank L. Ayala, L. Herr, C. R. McClung, S. Fraser and D. Batchelder for assistance in the early stages of this work, D. Hubbell and P. Wong for helpful suggestions, and T. Bisseling for antiserum against pea leghaemoglobin. Portions of this work were supported by USDA competitive grant 82-CRCR-1-1040, NSF grant PCM80-21906, US AID grant 144-R827, NIH grant 1 R01 GM34331-01, Hatch grant NE:146(MICLO 1473), and an all-university research initiation grant to F. B. D. and grants from ELF-Aquitane, l'Enterprise Miniere et Chimique, Rhone Poulenc Recherches et CDF Chimie, France to G.L.T. and a grant from the Netherlands Organization for Biological Research (BION) to F. G.

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