INFECTION OF THE TROPICAL LEGUME MACROPTILIUM ATROPURPUREUM
BY THE BROAD-HOST-RANGE RHIZOBIUM STRAIN ANU240

DECLARATION

The research in this thesis is my own work, except where acknowledgement is made, and has not been submitted for any other degree.

Thesis submitted for the degree of
Doctor of Philosophy
at the Australian National University

by

ROBERT WILLIAM RIDGE

March, 1985
DECLARATION

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ROBERT W. RIDGE
TO YUKO
PREFACE

The Figures for this thesis are provided in a second volume. Figures are formatted mostly in two or three parts. The upper part is referred to in the Figure legend as A, the next part as B, and then C in three-part Figures. Certain obvious cytological features are only rarely indicated in the Figures, even if mentioned in the Figure legends. For example mitochondria and rough endoplasmic reticulum (RER), which are well known cytological features. Light, fluorescence and transmission electron micrographs are provided with a bar scale at the lower right hand corner of each micrograph. Scanning electron micrographs are provided with a scale along the lower edge of each micrograph. All Figures are also supplied with their true magnifications. Tables referred to in the text are located at the end of their relevant Chapters in volume 1.
ACKNOWLEDGEMENTS

During any lengthy study, there are periods of success and failure, elation and disappointment, and even occasionally, some results. It is through such trials and tribulations that relationships are sealed. I would like to acknowledge first and foremost the friendship and loving companionship of Yuko Fukui, who after three years of these studies and two years of marriage still talks to me, and who has never stopped trying to teach me to be patient and to worry less. I wish her great success with her own post-graduate studies.

For friendship and many games of Daihinmin, my sincere thanks to the following for keeping soul and mind together (our bodies have been ruined): Toshi and Etsuko Suzaki, Teddy and Itsuko Maddess, Peter Hoeben and Joyce Mannaert, Roland, Inge and Sophie Stocker, and Suchai and Wipa Treerat.

During the course of these studies, my departmental colleagues have offered not only friendship, but also technical advice and assistance whenever asked, and I acknowledge especially Greg Bender, Jane Olsson, Jacek Plazinski and John Watson.

I acknowledge the following for their help and guidance during these studies:

Dr. Dietz Bauer, of the Charles F. Kettering Research Laboratory, Yellow Springs, Ohio, USA; for laboratory facilities from April to June, 1983; for demonstrating the basics of spot-inoculation; and for the kindness he and his family showed to me during my stay in Yellow Springs.
Dr. T.V. Bhuvaneswari, of the Charles F. Kettering Research Laboratory; for the use of her laboratory and equipment; for showing me how to find the zone of susceptibility in siratro; and for her kindness towards me during my stay in Yellow Springs.

Prof. Malcolm Brown, of the Botany Department, University of Texas at Austin, USA; for computer studies of a number of micrographs.

Dr. Adrian Gibbs, of the Virus Ecology Research Group, RSBS; for allowing me bench space in his laboratory during the latter half of 1982.

Prof. Bernard John, of Population Biology, RSBS; for allowing me bench space in his laboratory during the first half of 1983.

Mr. John Preston, of the Scanning Electron Microscopy Unit, Forestry, ANU; for demonstrating the use of the Cambridge Stereoscan and SEM preparation techniques.

Messrs. David Smith and David Sandilands, of the Computer Unit, RSBS; for making me feel like their only customer.

Mr. Suresh Tiwari, of the Department of Developmental Biology, RSBS; for demonstrating the freeze-substitution technique.

The Neurobiology Department, RSBS; for the use of their electrode puller and supply of glass electrodes; and for the use of their darkroom facilities to print the figures in this thesis.

Prof. John Pateman, Dr. John Watson, Dr. Kieran Scott and Dr. Murali Nayudu are thanked for reading and commenting on the manuscript.

I would also like to thank the Commonwealth Government of Australia for providing a post-graduate scholarship during these studies.
Finally, I would like to extend my appreciation and thanks to my two supervisors, Dr. Barry Rolfe and Dr. Peter Gresshoff. Special thanks to Dr. Rolfe for the provision of excellent light microscope equipment, the opportunity of a two-month visit to the Charles F. Kettering Research Laboratory in Yellow Springs, Ohio, and for his most enthusiastic approach to my studies.
ABBREVIATIONS

FM  Fluorescence Microscopy
LM  Light Microscopy
SEM Scanning Electron Microscopy
TEM Transmission Electron Microscopy
FITC Fluoroisothiocyanate
FS  Freeze-substitution

um  micron \((10^{-6} \text{ metres})\)
nm  nanometre \((10^{-9} \text{ metres})\)
ul  microlitre \((10^{-6} \text{ litres})\)
ug  microgram \((10^{-6} \text{ grams})\)

RT  Root Tip
SERH Smallest Emerging Root Hairs
DRHZ Developing Root Hair Zone
NRHZ No Root Hair Zone
MRHZ Mature Root Hair Zone
RH  Root Hair
-R  Minus Rhizobium

RER Rough Endoplasmic Reticulum
OPD Osmiophilic Droplets
pbm Peribacteroid membrane
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<td>Sym</td>
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<tr>
<td>Nod</td>
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<td>Fix</td>
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<td>Hae</td>
<td>Hair curling</td>
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<td>Transposon 5</td>
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<tr>
<td>kb</td>
<td>Kilo base pairs</td>
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<td>nif</td>
<td>Fixation genes</td>
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<td>Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>Kanamycin resistant</td>
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<td>Sp&lt;sup&gt;r&lt;/sup&gt;</td>
<td>Spectinomycin resistant</td>
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<tr>
<td>Sm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>Streptomycin resistant</td>
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<tr>
<td>HR</td>
<td>Hypersensitive Response (or Reaction)</td>
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<td>EPS</td>
<td>Exopolysaccharide</td>
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<td>PHB</td>
<td>Polyhydroxybutyrate</td>
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Restriction enzymes from:
- *Bacillus amyloliquefaciens H*
- *Escherichia coli BS5*
- *Haemophilus influenzae Rd*
- *Xanthomonas holcicola*
- *Caryophanon latum L*
ABSTRACT

The early events leading to the successful infection of the tropical legume *Macroptilium atropurpureum* (siratro) by the broad-host-range *Rhizobium ANU240* were studied. During these investigations, various mutant and transconjugant rhizobia were used as 'probes' of the infection process. Studies of the *Rhizobium* and plant surfaces were done using lectins specific for certain sugars.

It was found that nodulation of siratro occurs according to a pattern that relates to the position of the root tip at the time of inoculation. Root elongation studies showed that the total region of nodulation originates from a zone between the root tip and smallest emerging root hairs at time of inoculation, the point of maximum nodulation being approximately midway between these two points. By micro-inoculation of this midway point, study of the earliest infection events were greatly facilitated. Infection studies showed that the *Rhizobium* strain ANU240 affects and causes curling of emerging root hairs within 12 hours of inoculation. Between 12 and 20 hours the bacteria colonize the 'pocket' created by the curl. Also during this time the surface of the curled hair takes on a distinct brown appearance, as the outer hair wall thickens. Between 20 and 24 hours the rhizobia erode the hair wall matrix and enter an interfacial zone between the wall and host plasma membrane, pushing through the loosened hair wall microfibrils presumably by pressure from colony growth. Infection thread synthesis starts and ensues for 20-24 hours within the hair cell. From about 24 hours after inoculation, cell division commences in the cortex below the infection site. At about
48 hours after inoculation, the infection threads penetrate the lower wall of the hair cell and enter the dividing tissue beneath.

It was found that loss of the Symbiotic plasmid from the parent Rhizobium (to produce strain ANU265) not only caused loss of infecting capabilities of the Rhizobium, but also loss of erosion activities at the root surface previously unknown to occur. A single transposon inserted in the nodulation region of the Symbiotic plasmid (strain ANU1255) had a similar effect to complete loss of the symbiotic plasmid. However, a single transposon in a chromosomal site of one mutant (strain ANU2861) prevented bacterial entry into the hair cell at the plasma membrane in curled hairs in the infectible zone (between the root tip and smallest emerging hairs at time of inoculation). This demonstrated the possibility that bacterial contact at the host membrane is a major infection step. The host reacted to this mutant in a similar manner to phytopathogenesis. Another Rhizobium mutant, with a single transposon in the chromosome (strain ANU1260) was able to initiate nodule growth, but unable to properly release rhizobia into the host cell. Similarly, a transconjugant (strain ANU289(pJB5J1)) which contained the Sym plasmid from the pea nodulating Rhizobium leguminosarum, was also deficient in bacterial release. When a 14kb DNA fragment encoding the nodulation region of the Symbiotic plasmid of R. trifolii strain ANU843 was transferred to a Symbiotic plasmid-cured strain (to make transconjugant strain ANU845(pRt032)), it was able to initiate nodulation but was unable to effect normal bacterial release in white clover. All these bacterial release deficiencies appeared to be due to a lack of coordination between release and the host endogenous membrane system, which
produces the peribacteroid membrane. Thus, it was concluded that a
major infection step, where communication between host and
endosymbiont appear essential, occurs at the stage of bacterial
release. In subterranean clover, strain ANU845(pRt032) was able to
effect bacterial release, although bacteroid formation was defective.
This demonstrated the possible presence of host specific genes on this
region of the Symbiotic plasmid.

In lectin studies of the siratro root surface, the lectin RCA I was
found to attach to the tips of root hairs, suggesting the presence of
the sugar β-D-Galactose. If the infectible zone at a
micro-inoculation site was pre-incubated with the lectin, infection by
strain ANU240 was prevented. It is possible that the sugar β-D-
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'recognition sugar' that enables the rhizobia to recognize and attach
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CHAPTER 1

INTRODUCTION

The shoot-root junctions have been the focus of continuous research for almost a century (see Fred et al., 1932) and yet many of the definitive steps in the development of this symbiosis remain poorly understood. This is testimony to the experimental complexity encountered when studying bacterial-plant associations.

As early as 1957, McDow observed in roots of Vicia faba that nodulation by Rhizobium commenced with the formation of root hairs through the formation of a bacterial thread which grew into the cortex, thus initiating the nodule. In a careful study, McDow (1970) demonstrated that shortly after inoculation of seeding roots with nodulating Rhizobium, the root hairs showed strong deformation, typically, curling or branching responses, and in many cases root hairs that were infected were found. McDow observed that the bacteria were found after infection in a thread of tubular form continuity with the root cell walls. The thread originated with no apparent structural disruption of the root wall and appeared to have the same chemical composition as the root cell wall, insomuch as staining indication. The Elodinum within the infection thread wall were surrounded by a "normal matrix", which is probably a Rhizobium root-surface layer (Fred, 1970). The Elodinum within the infection-thread are extracellular (with respect to the host). In a search for symbiotic energy activity that might have been involved in the construction of the infection into the cell, McDow (1970) conducted no evidence to growth of Rhizobium on plant or cellulytic polysaccharides in culture. The lack of evidence of a chemical or physical penetration of the host cell wall has Akiyama (1960) to propose that the bacteria
1.1 THE STUDY OF RHIZOBIUM-LEGUME INFECTION

The Rhizobium-legume system has been studied continuously for almost a century (see Fred et al. 1932) and yet many of the rudimentary steps in the development of this symbiosis remain poorly understood. This is testimony to the experimental complexity encountered when studying bacterial-plant associations.

As early as 1887, Ward observed in roots of Vicia faba that nodulation by Rhizobium commenced with the infection of root hairs through the formation of a bacterial thread which grew into the cortex, thus initiating the nodule. In a careful study, McCoy (1932) demonstrated that shortly after inoculation of seedling roots with nodulating bacteria, the root hairs showed strong deformation, typically a curling or branching response, and it was among these root hairs that infections were found. McCoy observed that the bacteria were found after infection in a thread of tubular form continuous with the root cell walls. The thread originated with no apparent structural disruption of the hair wall and appeared to have the same chemical composition as the hair cell wall, inasmuch as staining indicated.

The Rhizobium within the infection thread wall were surrounded by a 'thread matrix', which is probably a Rhizobium polysaccharide (Dart 1977). The Rhizobium within the infection thread are extracellular (with respect to the host). In a search for hydrolytic enzyme activity that might have been involved in the penetration of the Rhizobium into the cell, McCoy (1932) could obtain no evidence for growth of Rhizobium on pectin or cellulosic polysaccharides in culture. The lack of evidence of a chemical or physical penetration of the hair cell wall led Nutman (1956) to propose that the bacteria
penetrated by a process similar to intussusception, intercalating into the root hair wall polymers, and initiating the thread as they contacted the plasmalemma. He proposed that a redirection of the site of active root hair tip growth by bacterial action resulted in an invagination of the hair wall back into the hair, forming the tubular wall structure of the infection thread. Subsequently, Fahraeus and Ljunggren (1959) reported that inoculation with infective bacteria resulted in an increase of constitutively produced polygalacturonase by the root itself. These authors interpreted their results as compatible with the invagination mechanism proposed by Nutman (1956). Ljunggren (1969) attempted to confront the problem presented by the inability of a loosely attached Rhizobium colony to generate an inward force greater than the pressure potential of the root hair cell; this would be required to initiate invagination at a locally softened region of cell wall. Ljunggren expressed the belief that Rhizobium enclosed by the induced deformation derived an advantage from the enclosed 'pocket'. The combination of Rhizobium multiplication, bacterial slime secretion, and wall softening induced within the pocket allowed expansion of the colony, ensheathed by the extending wall, with formation of an infection thread. Like McCoy (1932), Ljunggren believed that further thread growth involved the redirection of active root hair tip growth to the tip of the thread.

Efforts to repeat the pectinase assay of Fahraeus and Ljunggren (1959) using whole seedlings have failed (see reviews by Dart 1974, 1975, 1977). Using more sensitive techniques to detect polysaccharide degradation activity, Hubbell et al. (1978) reported pectolytic activity by Rhizobium colonies in culture. Subsequent work (Martinez-Molina et al. 1979) showed cellulase-degrading enzymes can also be
detected in *Rhizobium* cultures, thus re-establishing the possibility of polysaccharide-hydrolyzing enzymes of *Rhizobium* rather than host origin.

Direct observations of the initial events of infection have been sparse. Until 1980, ultrastructural observations were limited to three studies, all interpreting the infection process in support of the hypothesis of invagination. Studies by Sahlman and Fahraeus (1962) and Higashi (1966) preceded the availability of techniques suitable for adequate fixation and ultrastructural examination. In their electron microscopic study, Napoli and Hubbell (1975) properly interpreted the overall structure of sections of curled hairs. Their conclusions in favour of the invagination mechanism were based on illustrations which showed that the root hair cell wall was continuous with the infection thread wall and that no discontinuities were found in the hair wall. Some of their illustrations, however, suggested a degradation of the original hair cell wall at the point of transition and showed an inner layer of the hair cell wall which continued into the thread wall. The occurrence of such a cell wall change would be in conflict with their interpretation of the lack of physical penetration of the hair cell wall and would considerably alter the accepted view of the invagination mechanism of root hair infection. The questions raised regarding the feasibility of the invagination mechanism, the uncertain interpretation of the limited structural evidence, together with the observation of polysaccharide-hydrolyzing activity in *Rhizobium*, provided the impetus for Callaham and Torrey (1981) to re-examine the structural basis of root hair infection in the temperate legume, clover (*Trifolium repens*). Their results provided an alternative to the invagination hypothesis. Electron
micrographs of diverse infection sites showed in every case a
degradation of the root hair wall at the site of thread origin within
the curled hair enclosure. The thread wall was found to be a new
layer formed by the apposition of material by the host cytoplasm near
the penetrated wall. This new material surrounded the break,
encapsulating the invading rhizobia. They suggested that Rhizobium
enzymes are responsible for degradative penetration of the root hair
cell wall and that the localised concentration of hydrolytic enzymes,
as well as protection from cell lysis, is favoured by physical
constraints provided by the deformed root hair enclosures. Their
paper is a landmark in the cytological study of infection in the
Rhizobium-legume symbiosis, but was based on analysis of infected
tissues after the critical events of infection had occurred. A second
significant paper by Turgeon and Bauer (1982) described the time
course of early infection events in soybean (Glycine max) following
inoculation with Rhizobium japonicum. They found that bacteria became
attached to epidermal cells and root hairs within minutes of
inoculation, and marked root hair curling occurred within 12h.
Infection thread formation was visible under the light microscope
about 24h after inoculation and was observed in short, tightly-curled
hairs. These root hairs had not emerged from the epidermal surface
until after inoculation. By 48h the threads had progressed to the
base of the hair but had not yet penetrated the cortex. Increases in
cortical cell cytoplasmic volume and in mitotic division occurred in
advance of the penetrating infection threads. These studies by
Turgeon and Bauer were made possible for two reasons. Firstly, the
discovery by Bhuvaneswari et al. (1980) that the susceptibility of
soybean root cells to infections that generate nodules is transient;
and secondly, the development of a micro-inoculation technique (Turgeon and Bauer 1983) that was able to take advantage of the transient susceptibility to infection in soybean. Turgeon and Bauer’s paper (1982) thus represents the first attempt at observing infection events as they happened, rather than inferring these events from older material. Their paper, however, provides little cytological evidence at the electron microscopic level, and draws attention to the need to focus on those degradative events associated with the initiation of infection and colonization of the plant tissue by Rhizobium. Such events are presented and analysed in this thesis.

1.2 BAUER’S MODEL OF INFECTION

A model of infection proposed by Bauer (1981) incorporates the results of Turgeon and Bauer (1982) and is based on “what little is known of root hair structure and development” (Bauer 1981). The model suggests an intricate control of the synthesis of the Rhizobium enzymes that degrade root-hair wall structures. In light of the results presented in this thesis, it is remarkably intuitive. The model is summarized as follows:

The cell walls of root hairs appear to be composed of two distinct layers, called alpha (a) and beta (b) layers. The a layer is considered to be flexible while the b layer is relatively rigid. The a layer is continuous over the entire hair (see Fig. 1.1b). It appears to consist primarily of cellulose microfibrils randomly oriented in a plane parallel to the hair plasma membrane, with pectic polysaccharides and perhaps hemicelluloses forming an amorphous
matrix. The \text{b} layer consists primarily of cellulose microfibrils oriented in a plane parallel to the hair plasma membrane and oriented roughly parallel to the axis of the hair elongation. The \text{b} layer, in contrast to the \text{a} layer, does not extend over the entire hair (Fig. 1.1b), it develops inside the \text{a} layer, and extends as a cylinder almost to the hemispherical dome of the growing root hair tip.

Root hair cells develop from short epidermal cells called trichoblasts, which are formed by unequal epidermal cell divisions in the root meristem. Hairs develop only on epidermal cells that are still elongating and are initiated exclusively at the apical end of the epidermal cells. In Fig. 1.1a, a hair is emerging from the apical end of an epidermal cell. The flexible \text{a} layer tip of the hair bulges outward as a result of turgor pressure against an area of localised removal of the rigid \text{b} layer. The localised removal of the \text{b} layer is presumed to be a consequence of host-induced disintegration of the microfibrillar matrix or inhibition of \text{b} layer synthesis. It is also presumed that deposition of new \text{a} layer material is heaviest at the apex of the hemispherical swelling. As a consequence, any bacterial cell attached to the emerging tip will gradually be displaced from the apex to the edge of the hemisphere (Fig.1.1b). The model proposes that curling results from a localised inhibition of the \text{b} layer deposition in the emerging hair, induced by an attached \text{Rhizobium} cell. The rigid cylinder of \text{b} layer material thus does not develop past the attached \text{Rhizobium} cell but continues to be deposited inside the \text{a} layer opposite the attached \text{Rhizobium} (Fig. 1.1c). As the hair continues to elongate, the flexible hemispherical tip gradually pivots around the attached bacterium, resulting in a tight curl that envelops the \text{Rhizobium} (Fig. 1.1d,e).
Recently, studies on pea root hair walls have revealed that the ultrastructure of the developing hair cell wall is opposite to the arrangement in non-legume root hairs (Goosen de Roo et al. 1984). The fibrillar \( b \) layer develops not along the plasma membrane but at the outside of the cell. The authors presume the fibrillar structure is a network in a right-angular arrangement. These results may mean that Bauer's model will need adjustment, but until electron micrographs of these results are published the question should be left open. Certainly the external position of the \( b \) layer may help explain more easily why Rhizobium is able to curl hairs. If the rigid \( b \) layer alone is affected by the rhizobia, the \( a \) layer would presumably still prevent explosion of the hair cytoplasm. This may not be the case if the \( b \) layer is on the inside of the hair cell, where the \( a \) layer first has to be eroded (or otherwise affected).

Nevertheless, the \( b \) layer model and the results of Bhuvaneswari et al. (Bauer et al. 1980; Bhuvaneswari et al. 1980) suggest that the first interactions critical to the infection process in plants such as soybean most probably take place at the apical ends of short epidermal cells, cells that have completed most of their axial elongation but have not produced a long hair.

1.3 OTHER KINDS OF INFECTION

Although work on infection has concentrated on root hair curling and consequent infection thread synthesis, other kinds of infection are known. In legumes such as peanut (Arachis hypogaea) and Stylosanthes (eg Stylosanthes hamata), infection threads are not found in the nodules (see Dart 1977). The only publications on infection of such
legumes are those of Chandler (1978) and Chandler et al. (1982) which describe the infections of peanut and Stylosanthes respectively. Root nodules of these legumes occur only at lateral root junctions and results from direct invasion by rhizobia through spaces between epidermal cells. Invasion of the host cortical cells occurs through structurally altered cell walls. The bacteria reach the site of nodule initiation in the root cortex by progressive collapse of the initially invaded cells, which are compressed by neighbouring cells to form intercellular thread-like infection zones (the author suggests that this resembles the defence mechanisms of some plants to invasion by pathogenic organisms, citing Tarr (1972)). The bacteria multiply in the invaded cells of the nodule initial which divide repeatedly to form the nodule. Bacteroids form only when the host cells cease to divide. Although there are minor differences between peanut and Stylosanthes in infection (for example origin of nodule initials) the basic principles described by Chandler are the same.

Another kind of infection is that of stem nodulation, known to occur in Neptuna oleracea, Aeschynomene indica, and Sesbania rostrata. Duhoux (1984) describes stem nodules of S. rostrata having a large central mass of infected cells. Stem 'mamillae' (points of quiescent adventitious roots) are regularly arranged in vertical files along the stem and develop into nodules when they are infected by a specific Rhizobium. Each nodule arises from the development of an infected region of the incipient root cortex. The infection in S. rostrata has been shown to proceed in four sequential stages (Duhoux 1984).

According to Duhoux, some of them have never been shown to occur in other legumes: (i) bacterial penetration takes place in degenerated (dead) cortical cells; (ii) proliferation of the bacteria occurs in
the intercellular cavities and initiates a meristematic nodule; (iii) protrusion of infection threads at first occurs intercellularly and then intracellularly from the cavities; (iv) finally there is an intracellular release of rhizobia by an endocytotic process. Despite Duhoux's declaration that some of these stages have never been shown to occur in other legumes, they appear to be very similar to those stages described by Chandler (1978) and Chandler et al. (1982), for root nodules of peanut and Stylosanthes respectively. It is confusing that even though Duhoux cites Chandler's work in his paper, he does not appear to grasp the connection.
1.4 Rhizobium—LEGUME VS PATHOGEN-HOST

"Far from being an insurmountable obstacle to the analysis of an organic system, a pathological disorder is often the key to understanding it"

Konrad Lorenz (1903- )

Rhizobium-legume infection and nodulation bear a striking resemblance to infection and disease induction by plant pathogenic organisms (Vance 1983). However, it is intriguing that no bacterial pathogens have evolved the fine precision that characterizes the interactions of Rhizobium with their hosts. Earlier concepts suggesting that nodule formation and symbiosis did not evolve from a primitive pathogenic interaction may need re-evaluation (Parker 1957; Dilworth and Parker 1969). According to Vance (1983), however, irrespective of the final manifestation of the Rhizobium-legume interaction, it has many features in common with plant-parasite infection and development. These features include:

a) Binding of the microbes to the host plant
b) Penetration into the host plant
c) Host plant response to penetration
d) Redirection of host plant metabolism
e) Morphological manifestation of the interaction ie nodules and symptoms

Approaching the Rhizobium-legume association from a phytopathological perspective reveals the limitations of our understanding, and may offer novel approaches to further research (see Vance 1983).
There are many stages during the infection process at which interaction between plant and symbiont may result in acceptance or rejection. Heath (1974) refers to such stages (in the context of plant pathology) as 'switching points', where the outcome of the interaction determines the subsequent progress of the infection. Thus, successful infection depends upon the 'correct' response at every stage, while rejection requires an 'incorrect' response at only one stage. An incorrect response could be interpreted as the triggering of an active defence mechanism or the failure to induce acceptance. It can be expected that each of the switching points represents a major gene, especially in the light of microscopical work by Ellingboe (1972) who has shown that different genes for resistance to wheat and barley mildew in their respective hosts are manifested at different points and in different ways during the infection process; and from the studies of Littlefield (1973), who has shown a similar situation in the resistance of flax to Melampsora lini. The studies presented in this thesis provide evidence for such switching points in the context of the Rhizobium-legume symbiosis, and emphasize the important role of structural studies in pinpointing the precise site within the tissue or cell of the host where the critical interaction occurs, leading to the triggering (or not) of acceptance or rejection mechanisms. They also emphasize the importance of using Rhizobium mutants and transconjugants as probes of the infection process, especially when the genes involved have been precisely located.
All stages between symbiotic partners may contain specific interactions possible only between homologous partners. However, if such specific interactions, which contain an element of recognition, are part of an essential step, their failure will prohibit all further stages. This failure will be most dramatic if it occurs early in the total sequence of interactions. These interactions form a very subtle, intricate pattern, operating mainly at a subcellular level. This makes their analysis very difficult. Much information may be derived from comparisons with deviations in the normal development, when less compatible combinations are studied. The progress in the microbial genetics of Rhizobium has markedly improved possibilities for such comparisons and enabled a further analysis of the interactions during infection and nodulation. Evidence indicating that both the plant and the bacterial genomes can be easily modified to either enhance or reduce the symbiotic association provides strategies for reducing constraints to biological nitrogen fixation (Hooykaas et al. 1981; Roughley et al. 1981; Long et al. 1982; Scott et al. 1982; Carroll et al. 1985; McNeil et al. 1985).

Krasilnikov first reported (in 1941) the transfer of host range between Rhizobium species (cited in Beringer et al. 1980). Transformation of auxotrophic mutants and extension of nodulation host range in R. japonicum (Balassa 1956) and R. lupini (Balassa 1960) has been reported. Transduction, both specialized and generalized, has been observed in R. meliloti and R. leguminosarum (reviewed by Beringer et al. 1980). Conjugation was first reported in R. lupini (Heumann,
1968) and a circular linkage map was established by means of this naturally-occurring conjugal system. The genetic analysis of Rhizobium was however, hindered by the absence of genetically well characterized symbiotic mutations.

1.5.1 PLASMID INVOLVEMENT IN THE SYMBIOSIS
The lack of segregation of nodulation and host range genes among recombinant Rhizobium has suggested that these genes were not chromosomally located (Johnston and Beringer 1977). Higashi (1967) presented data that the nodulation genes were plasmid-borne, as treatment with acridine orange, a known plasmid-curing agent, rendered the Rhizobium nodulation defective. More recent work (e.g. Zurkowski and Lorkiewicz 1978, 1979; Nuti et al. 1979; Zurkowski 1980; Rolfe et al. 1980a; Djordjevic et al. 1982; Kondorosi et al. 1982; Djordjevic 1983; Djordjevic et al. 1984; Kondorosi et al. 1984; Morrison 1984; Rolfe & Shine 1984) has shown that the 'Sym' plasmid carries not only nodulation genes, but host range and nitrogen fixation genes.

1.5.2 TRANSPOSONS AS MUTAGENS FOR THE STUDY OF THE SYMBIOSIS
All the mutants used in this thesis were initially isolated using transposon mutagenesis. Transposons are discrete DNA entities which are able to translocate from one location to another in DNA, in the absence of host-mediated recombination functions, at varying frequency and with varying specificity. Moreover, they usually carry an antibiotic resistance gene (reviewed by Kleckner 1981). As mutagens, transposons offer the unique advantage of allowing positive selection for an insertionally
inactivated gene. In contrast, point mutations, induced by chemical
mutagens or UV irradiation, can in general, only be detected after
screening survivors. The transposon Tn5 encodes resistance to
aminoglycoside antibiotics (kanamycin and neomycin), transposes at low
frequencies with little specificity and results in polar inactivation
of downstream genes of an operon (Berg et al. 1980, reviewed by Berg
and Berg, 1983).

The conjugative broad-host-range IncP-1 group plasmids (Datta et al. 1971) are readily transmissible to Rhizobium by conjugation (Datta et al. 1971; Beringer 1974) or transformation (Dunican and Tierney 1973). Broad-host-range plasmids of other incompatibility groups, such as the IncQ plasmids (Bagdasarian et al. 1981), can also be introduced into Rhizobium. Bacteriophage Mu, a transposable element used in E.coli
genetics, is unstable in the Rhizobiaceae genus Agrobacterium (van Vliet et al. 1978). Phage Mu can by introduced into Rhizobium via the IncP-1 conjugative plasmids but is unstable (Boucher et al. 1977; van Vliet et al. 1978).

Since plasmids containing phage Mu are unstable in Rhizobium, an
efficient mutagenesis system has been designed in which the transposon
Tn5 is 'loaded' onto an unstable RP4::Mu plasmid. This provides an
effective delivery system for the transposon due to the instability of
the (suicide) vector (Beringer et al. 1978), and results in efficient
Tn5 mutagenesis of R.leguminosarum.

Such a technique allows for positive selection of mutants and, when
coupled with rapid plant assays (Rolfe et al. 1980b), facilitates the
screening of large numbers of mutated bacteria for symbiotic defects.
Due to its low DNA sequence insertional specificity (Berg and Berg
1983) and the fact that its aminoglycoside antibiotic resistance
determinants are easily selected for in *Rhizobium*, Tn5 has been the transposon most frequently used. Other transposons, Tn7 (Bolton *et al.* 1984) and Tn1 (Casadesus *et al.* 1980) for example, have unique or limited sites of insertion in the *R. meliloti* Sym plasmid. The antibiotic marker (Kmr) acts as a genetic 'flag' for the mutated locus and the lack of certain restriction endonuclease sites within Tn5 (Jorgensen *et al.* 1979) allows DNA restriction fragments containing Tn5 to be readily isolated by molecular cloning. Such procedures facilitate the isolation of corresponding wild-type restriction fragments from DNA libraries (Scott *et al.* 1982). These fragments can be used to demonstrate that the transposon is responsible for the genetic defect (one of the disadvantages of chemical mutagenesis) and allow direct analysis of the gene at the DNA level (ibid).

1.6 AIMS AND INVESTIGATIONS

As indicated in section 1.1, higher plant-bacterial interactions are known almost exclusively in terms of post-infection events. The pre-initiation events remain largely obscure and inaccessible. If the plant-bacterial interaction is to be manipulated, with control of the undesirable and promotion of the desirable consequences as its objective, at least a working understanding of pre-infection events is a necessary prerequisite. It is fortunate that development in *Rhizobium* genetics has reached the stage where mutant and transconjugant strains can be made. By using such strains, infection events can be isolated, and using *Rhizobium* transconjugants, correlated with particular *Rhizobium* genes.
1.6.1 PILOT STUDIES

During the early part of these studies a number of Rhizobium-plant 'systems' were investigated. These included clovers (Trifolium sp.), siratro (Macroptilium atropurpureum), peanut (Arachis hypogaea), Aeschynomene indica and Sesbania rostrata. In addition, a large number of auxotrophic and prototrophic mutants (Tn5-induced) and transconjugants carrying a transmissible Sym plasmid (from strain ANU240) were tested on siratro.

1.6.2 DECISIONS BASED ON PILOT STUDIES

The strain ANU240-siratro system was found to be the best for the following reasons:

(i) The plant was suspected at that time (confirmed later) to have a zone of susceptibility similar to that of soybean, which is infected only in emerging root hairs, not mature hairs. This is of considerable advantage in localising infection, essential when examining the very early infection events.

(ii) The plant seed is in good supply, has a hard seed coat that is easy to sterilize, is an ideal size for the pouch method, and will germinate easily with high efficiency.

(iii) The Rhizobium strain ANU240 is a fast-growing, broad-host-range bacterium and was proving at that time to be very promising for the supply of mutants and transconjugants for planned genetic/infection biology correlative work.
(iv) The ultrastructural details of infection and the exact timing of these events under controlled conditions had yet to be presented in any publication in a tropical plant system, the nearest being work on soybean by Turgeon & Bauer (1982) of which most was at the light microscope level.

(v) Studies of this tropical system would complement studies being done in the temperate (clover) system.

(vi) The plants could be grown under strictly controlled conditions in a seed pouch.

Thus, the strain ANU240-siratro system is useful not only for the study of infection events, but by manipulation of the more genetically tractable fast-growing strain ANU240, such events can be correlated with Rhizobium genes.

A number of mutant and constructed Rhizobium strains were reserved for further studies on siratro. These were:

Strain ANU265 a Sym plasmid-cured spectinomycin-resistant derivative of strain ANU240 (see Chapter 9)

Strain ANU1255 a strain ANU240 derivative carrying a single insert Tn5 in the nodulation region of the Sym plasmid, Hac¯ Nod¯ phenotype (see Chapter 9)

Strain ANU1260 a strain ANU240 derivative carrying a single insert Tn5 in the chromosome causing a bacterial release mutation (Bar¯) (see Chapter 12)
Other strains isolated (or constructed) during the thesis studies, were:

Strain ANU845(pRt032)  a Sym plasmid-cured derivative of the _B. trifolii_ strain ANU843 carrying the 14kb HindIII fragment of the Nod region of the Sym plasmid of strain ANU843 (see Chapter 12)

Strain ANU2861  an Ade$^-$ derivative of strain ANU240 carrying a single Tn5 in the chromosome causing a Hac$^+$ Nod$^-$ phenotype (see Chapter 11)

Strain ANU265(H1)  a Sym plasmid-cured derivative of strain ANU240 carrying a 6.7kb HindIII fragment of the Nod region of the Sym plasmid (see Chapter 10)

Strain ANU265(XA1)  a Sym plasmid-cured derivative of strain ANU240 carrying a 16kb XhoI fragment of the Nod region of the Sym plasmid (see Chapter 10)

In addition, embedded nodules of siratro that had been infected with transconjugant strain ANU289(pJB5JI) were examined (see Chapter 12).

1.6.3. MAJOR AIMS: ANALYSIS OF THE SYMBIOTIC SEQUENCE

1. Investigate the infection of the tropical legume siratro by the _Rhizobium_ strain ANU240,

2. Deduce the steps involved in this infection,

3. Apply various mutants and transconjugants to the system as probes of the infection process and correlate particular bacterial genes with infection biology.
A phenotypic key devised by Vincent (1980) and Rolfe et al. (1981) to describe the stages of the infection process has proved a useful guide during these studies. A summary of the known or inferred steps of infection at the time of commencement of this study is given in Table 1.

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Multiplication on root surface (initial phase)</td>
</tr>
<tr>
<td>2.</td>
<td>Attachment to root surface (root colonization)</td>
</tr>
<tr>
<td>3.</td>
<td>Branching and root hairs (root colonization)</td>
</tr>
<tr>
<td>4.</td>
<td>Formation of cortical infection thread</td>
</tr>
</tbody>
</table>

Table 1: Summary of the Known or Inferred Steps of Infection at the Time of Commencement of This Study.
<table>
<thead>
<tr>
<th>Stage</th>
<th>Abridged description</th>
<th>Phenotypic code</th>
</tr>
</thead>
<tbody>
<tr>
<td>I Preinfection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Multiplication on root surface ('rhizoplane')</td>
<td>Root colonization</td>
<td>Roc</td>
</tr>
<tr>
<td>2. Attachment to root surface</td>
<td>Root adhesion</td>
<td>Roa</td>
</tr>
<tr>
<td>3. Branching of root hairs</td>
<td>Hair branching</td>
<td>Hab</td>
</tr>
<tr>
<td>4. 'Marked' curling of root hairs</td>
<td>Hair curling</td>
<td>Hac</td>
</tr>
<tr>
<td>II Infection and Nodule Formation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. Formation of infection thread</td>
<td>Infection</td>
<td>Inf</td>
</tr>
<tr>
<td>6. Development of polyplloid (disomatic) meristem; nodule development and differentiation</td>
<td>Nodule initiation</td>
<td>Noi</td>
</tr>
<tr>
<td>7. 'Intracellular' release of Rhizobium from infection thread</td>
<td>Bacterial release</td>
<td>Bar</td>
</tr>
<tr>
<td>8. 'Intracellular' multiplication of Rhizobium and development of full bacteroid form</td>
<td>Bacteroid development</td>
<td>Bad</td>
</tr>
<tr>
<td>III Nodule Function</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9. Reduction of N$_\text{2}$ to NH$_4^+$ (nitrogenase)</td>
<td>Nitrogen fixation</td>
<td>Nif</td>
</tr>
<tr>
<td>10. Complementary biochemical and physiological functions</td>
<td>Complementary functions</td>
<td>Cof</td>
</tr>
<tr>
<td>11. Persistence of nodule function</td>
<td>Nodule persistence</td>
<td>Nop</td>
</tr>
</tbody>
</table>
CHAPTER 2

MATERIALS AND METHODS
2.1. BACTERIOLOGICAL MEDIA

BERGERSEN'S MODIFIED MEDIUM (BMM) (Bergersen 1961)

- Na\(_2\)HPO\(_4\)•12H\(_2\)O: 360 mg\(\cdot\)l\(^{-1}\)
- MgSO\(_4\)•7H\(_2\)O: 80 mg\(\cdot\)l\(^{-1}\)
- FeCl\(_3\): 3 mg\(\cdot\)l\(^{-1}\)
- CaCl\(_2\): 40 mg\(\cdot\)l\(^{-1}\)
- Mannitol: 3 g\(\cdot\)l\(^{-1}\)
- Thiamine: 2 mg\(\cdot\)l\(^{-1}\)
- Biotin: 0.2 mg\(\cdot\)l\(^{-1}\)
- Sodium glutamate: 500 mg\(\cdot\)l\(^{-1}\)
- Yeast extract: 500 mg\(\cdot\)l\(^{-1}\)
- Gamborg's trace elements: 1 ml\(\cdot\)l\(^{-1}\)
- pH 7

TRYPTONE YEAST MEDIUM (TY) (Beringer 1974)

- Bacto-tryptone: 5 g\(\cdot\)l\(^{-1}\)
- Yeast extract: 3 g\(\cdot\)l\(^{-1}\)
- CaCl\(_2\)•2H\(_2\)O: 0.9 g\(\cdot\)l\(^{-1}\)
- pH 6.8

FAHRAEUS' MEDIUM (F) (modified) (Vincent 1970)

- CaCl\(_2\)•2H\(_2\)O: 100 mg\(\cdot\)l\(^{-1}\)
- MgSO\(_4\)•7H\(_2\)O: 120 mg\(\cdot\)l\(^{-1}\)
- KH\(_2\)PO\(_4\): 100 mg\(\cdot\)l\(^{-1}\)
- Na\(_2\)HPO\(_4\)•12H\(_2\)O: 150 mg\(\cdot\)l\(^{-1}\)
- Ferric citrate: 1.5 mg\(\cdot\)l\(^{-1}\)
- Gibson's trace elements: 1 ml\(\cdot\)l\(^{-1}\)
- pH 6.5
**JENSEN'S MEDIUM (modified)**

<table>
<thead>
<tr>
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</tr>
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<tbody>
<tr>
<td>(KH_2PO_4)</td>
<td>1 g. l(^{-1})</td>
</tr>
<tr>
<td>(K_2HPO_4)</td>
<td>0.2 g. l(^{-1})</td>
</tr>
<tr>
<td>(MgSO_4 \cdot 7H_2O)</td>
<td>0.2 g. l(^{-1})</td>
</tr>
<tr>
<td>(NaCl)</td>
<td>0.2 g. l(^{-1})</td>
</tr>
<tr>
<td>(FeCl_3)</td>
<td>0.1 g. l(^{-1})</td>
</tr>
<tr>
<td>Gibson's trace elements</td>
<td>0.1 ml. l(^{-1})</td>
</tr>
<tr>
<td>pH</td>
<td>6.8</td>
</tr>
</tbody>
</table>

**GAMBORG'S TRACE ELEMENT SOLUTION (Gamborg and Eveleigh 1968)**

<table>
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<tbody>
<tr>
<td>(MnSO_4)</td>
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<tr>
<td>(H_3B_0_3)</td>
<td>3 g. l(^{-1})</td>
</tr>
<tr>
<td>(ZnSO_4 \cdot 7H_2O)</td>
<td>3 g. l(^{-1})</td>
</tr>
<tr>
<td>(Na_2MoO_4 \cdot 2H_2O)</td>
<td>250 mg. l(^{-1})</td>
</tr>
<tr>
<td>(CuSO_4 \cdot 5H_2O)</td>
<td>250 mg. l(^{-1})</td>
</tr>
<tr>
<td>(CoCl_2 \cdot 6H_2O)</td>
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**GIBSON'S TRACE ELEMENT SOLUTION (Gibson 1963)**

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<td>(H_3B_0_3)</td>
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<tr>
<td>(ZnSO_4 \cdot 7H_2O)</td>
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<tr>
<td>(CuSO_4 \cdot 5H_2O)</td>
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<tr>
<td>(H_2MoO_4 \cdot H_2O)</td>
<td>90 mg. l(^{-1})</td>
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### 2.2 ANTIBIOTICS

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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
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<td>ANU845(pRt032)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>100</td>
</tr>
</tbody>
</table>

Km = Kanamycin  
Sm = Streptomycin  
Sp = Spectinomycin  
Rif = Rifampicin  
Cb = Carbenicillin

### 2.3 RHIZOBIUM CULTURE

All stock cultures were maintained on BMM slopes at 4°C and also as frozen cultures in 20% glycerol at -20°C. When growing rhizobia from stock, cultures were first streaked onto fresh BMM plates rather than directly onto TY because the high yeast content of this media has a 'bloating' effect on the bacteria when coming out of the frozen state;
the mannitol in BMM enables the culture to recover quickly. Cultures were checked for their selective characteristics monthly and subcultures from the original frozen stocks were made every six months. In addition, all strains were checked for their various phenotypes on plants at every experiment or every three months.

2.3.1 RHIZOBIUM CULTURE FOR POUCH ASSAY

Stock cultures were streaked onto selective media and single colonies were used to inoculate 30ml of BMM in 125ml culture flasks. These starter cultures were maintained on a rotary shaker (200rpm) at 30°C until late log/early stationary phase (24 hours for ANU240). The optical density of this culture was determined (Klett 420 for ANU240) and a volume equivalent to 1ml of a Klett 200 suspension was added to 50ml of fresh BMM in a 250ml culture flask, with additions of relevant antibiotics where necessary. Inoculum cultures were grown to late log/early stationary phase in the same manner as the starter cultures and used undiluted (approx. 10⁹ cells ml⁻¹) for both flood and spot inoculation. Strain characteristics are given at the beginning of relevant chapters.

2.3.2 RHIZOBIUM CULTURE FOR PLATE ASSAY

Stock cultures were streaked onto selective TY media and single colonies were streaked directly on to one half of the F media plates for rapid plate assay, before seedlings were 'planted' on the agar surface.
2.4 SEED GERMINATION

2.4.1 SIRATRO
Siratro seeds were etched for 15 minutes with concentrated $\text{H}_2\text{SO}_4$, washed thoroughly with tap water, surface sterilized with 12% HOCl for 10 minutes and washed with sterile distilled water 5 times. The seeds were left to soak for 30 minutes before germinating over two days in plates of BMM in 1.5% agar at 30°C. No contaminants were detected with the BMM and germination was >90%. Seeds were also stored after sterilization in BMM plates for up to two weeks with no consequent loss of germination rate or increase in contamination upon incubation. Longer periods of storage gave lower germination rates.

2.4.2 WHITE AND SUBTERRANEAN CLOVER
Clover seeds were soaked in distilled water for 15 minutes, rinsed in 75% ethanol for 5 min. and washed 3 times with sterile distilled water. The seeds were then transferred to 5% hypochlorite for 15 min. and washed 5 times with sterile distilled water. They were germinated over two days in plates of BMM in 1.5% agar at 25°C or stored at 4°C for up to two weeks. Contamination occurred at a rate of 3%-5% when incubated for germination, the rapid growth of such contaminants (which were not Rhizobium) on BMM allowed easy visibility and avoidance of seeds in those areas of the plate.

2.5 GROWTH OF SEEDLINGS

2.5.1 POUCH METHOD
Germinated siratro seeds with radicles 1-2 cm long were transferred aseptically to plastic growth pouches (Northrup, King & Co., Minneapolis, Minnesota, U.S.A.) which had been previously watered with 7.5 ml of half strength Jensen's medium, 3 plants per pouch. The quantity of liquid medium was critical as the seedlings did not grow well in over-wet conditions. Seedlings were maintained in a Conviron growth chamber at 85% RH; 29°C day (13 hours); 26°C night; photon flux density 200-220 umol m⁻² s⁻¹ at midday from Sylvania Gro-lux VHO cool white fluorescent tubes (temperature and lighting were brought in and out gradually over 3-hour periods). The pouches were kept upright (roots kept dark with aluminium foil) and checked daily for watering requirements (Figs. 2.1 and 2.2).

Different strains under test were separated (Fig. 2.1). Minus-Rhizobium controls were watered last so that any contamination caused by handling techniques would be observed. No nodulation or hair curling was ever observed in these controls. Thus, although the pouch method is not aseptic in the true sense - because of the open top of the pouch - careful use of controls monitored possible handling errors. Wild-type controls always nodulated around the RT mark (see Chapter 4).

Pouches were used as supplied without autoclaving, as this badly wrinkled the pouch. However, a number of pouches were gamma-irradiated by Ansell (Melbourne) and used in some experiments. These too were never contaminated.

2.5.2 PLATE METHOD
Techniques followed Rolfe et al. (1980). For white clover, 4-6 seedlings per plate, for subterranean clover and siratro, 2 seedlings
per plate. Seedlings were placed with the radicle on the streaked half of an F medium plate. The plates were sealed with Nescofilm (Nippon Shoji Kaisha Ltd., Osaka, Japan) and a number of holes punched in the film at the 'top' of the plate to allow gas exchange. The plants grew well under such conditions but the nature of inoculation meant that nodulation was slower than pouched plants. Siratro was grown under the same conditions as in section 2.5.1 above. Clovers were grown at 22°C day (16hr), 18°C night, photon flux density 200-250 umol m\(^{-2}\) s\(^{-1}\) at midday from Sylvania Gro-lux VHO cool white fluorescent tubes. Lighting and temperature changes were not graduated but temperature changes took about one hour to balance out.

2.6 MICROSCOPY

For all microscopic work, as many specimens were prepared as possible, but in all experiments at least ten specimens per treatment were prepared and examined. All presented results are representative examples of these examined specimens.

2.6.1 SCANNING ELECTRON MICROSCOPY (SEM)

Three methods were used for specimen preparation.
1. Specimens were frozen onto the exposed surface of a metal block standing in liquid nitrogen, transferred to the cold stage (-40°C) of a freeze-drier (Dynavac L410M) and dried under vacuum at -60°C for 8 hours. Specimens were gradually brought to room temperature before the vacuum was released, mounted on stubs with double-sided sticky tape, coated with approx. 200 Angstroms gold (Dynavac vacuum coater CE12/14S) and viewed on a Cambridge Stereoscan S180 at 30kV.
2. Use of freeze-substitution technique. Specimens were frozen by plunging into liquid propane cooled with liquid nitrogen, transferred in small plastic containers to vials of 1% OsO₄ in dry acetone over molecular sieve (at the same temperature) and then left for 3 days at -80°C. The acetone melted within an hour at this temperature and the specimens immersed. After substitution, specimens (now black from the OsO₄) were gradually brought to room temperature over 24 hours, rinsed in fresh dry acetone and critical-point-dried in CO₂ (Balzers CPD 010). Specimens were mounted, coated and viewed as described above. This treatment produced much better results than (1).

3. Na-ethoxide technique - basically follows Pring (1975) and enables three-dimensional views of the infection site and threads. Embedded material (see following section) was sectioned to the centre of an infection site and Na-ethoxide was dripped onto the cut surface for 1 minute, the block was washed with EtOH, then water, then EtOH again, this prevented any crystalline deposit. The specimens were mounted, coated and viewed as described above. It is possible to view another part of the same block simply by re-cutting and repeating the procedure.

2.6.2 TRANSMISSION ELECTRON MICROSCOPY (TEM)

GLUTARALDEHYDE/PARAFORMALDEHYDE FIXATIVE

1) to 5ml H₂O, add 0.5g paraformaldehyde
2) heat to 60°C and add drops of 0.1M NaOH until the paraformaldehyde dissolves
3) add: 2.5ml of 25% EM grade glutaraldehyde
   12.5ml of 0.05M phosphate buffer at pH 6.8
   5ml H₂O
Makes 25ml of 2.5% glutaraldehyde/2% paraformaldehyde in 0.025M phosphate buffer at pH 6.8-7.0

**OSMIUM TETROXIDE FIXATIVE**

1g of OsO$_4$ was dissolved by stirring in 50ml of distilled water to make a 2% solution.

The above highly toxic fixatives were always used in the fume cupboard, and great care was taken to avoid exposure of laboratory personnel to their vapours. Paraformaldehyde is particularly difficult to handle because of its very fine powder form. The glutaraldehyde/paraformaldehyde fixative could be stored for up to two weeks at 4°C. Osmium fixative was always used freshly prepared prior to use.

**REYNOLDS' LEAD CITRATE** (Reynolds 1963)

- Pb(NO$_3$)$_2$: 1.33g
- Sodium citrate: 1.76g
- CO$_2$-free H$_2$O: 30ml
- pH 12

**TOLUIDINE BLUE**

- Toluidine blue: 0.5g
- Na$_2$CO$_3$: 0.1g
- H$_2$O: 100ml
- pH 11.1

**PHOSPHATE BUFFER**

To make 0.05M phosphate buffer
Solution A: 0.87g $K_2HPO_4$ in 50ml distilled $H_2O$
Solution B: 0.90g $KH_2PO_4$ in 66.6ml distilled $H_2O$
Add 49ml of A and 51ml of B to 100ml of distilled $H_2O$

makes 200ml of buffer

SPURR'S RESIN (Spurr, 1969)

Vinyl cyclohexene dioxiae 10g
Diglycidyl ether of polypropylene glycol 6g
Nonenyl succinic anyhydride 26g
Dimethylamino ethanol 0.4g

Stirred for 5 min.

Pot life is 2 days or it can be stored at -20°C for several weeks.
Spurr's resin is highly toxic in liquid form but is safe once polymerized, though care was taken to avoid sawdust when trimming blocks by sawing in a suction cabinet.

Specimens were routinely fixed in 2.5% glutaraldehyde/2.0% paraformaldehyde in 25mM $PO_4$ buffer at pH 6.8 for 6 hours, post-fixed (after 3 rinses in buffer) in 2% $OsO_4$ in distilled water for 2 hours, rinsed and dehydrated in a graduated acetone series. In the literature, fixation schedules for legume roots are exampled as follows: Bassett et al. (1977) - 3% glutaraldehyde in 0.025M $PO_4$ buffer (pH7.2) post-fixed with 2% $OsO_4$ in the same buffer; Newcomb et al. (1977) - 2.5% glutaraldehyde in 0.025M $PO_4$ buffer (pH6.8) post-fixed with 1% $OsO_4$ in the same buffer; Werner et al. (1980) - 3% glutaraldehyde in 0.1M Sörensen's buffer (pH7) post-fixed with 2% $OsO_4$ in the same buffer; Turgeon and Bauer (1982) - 4% glutaraldehyde in 0.05M $PO_4$ buffer (pH7.2) post-fixed with 3% $OsO_4$ in the same buffer;
Hirsch et al. (1983) - 3.5% glutaraldehyde/1% paraformaldehyde in 0.025M or 0.05M cacodylate buffer post-fixed with aqueous 1% OsO₄. The schedule used during the thesis work is one recommended by O'Brien and McCully (1981) for plant tissue. From the results it is clear that this fixation is adequate for some parts of the root tissue but not for others. After fixation and dehydration, specimens were infiltrated with Spurr's resin gradually over 3 days and embedded in fresh resin overnight at 60°C. Freeze-substituted specimens for TEM (see SEM) were rinsed three times in fresh dry acetone, infiltrated in Spurr's resin gradually over two weeks, and then embedded. This was done to ensure proper infiltration of the inner parts of the material, which is particularly impermeable due to ice crystal formation.

Sections for light microscopy were cut at 0.5μm with a glass knife and for electron microscopy with a Dupont diamond knife at approx. 70nm (silver sections) on a Reichert-Jung Ultracut OMU4. TEM sections were mounted on 0.3% formvar on slot grids and stained with a saturated solution of uranyl acetate in 50% methanol for 15 minutes and Reynolds' lead citrate for 10 minutes with thorough washings after each stain. They were then viewed with either an Hitachi H500 (at 75kv) or H600 microscope (80kV).

2.6.3 LIGHT (LM) AND FLUORESCENCE MICROSCOPY (FM)

LM sections were stained with toluidine blue at pH 11.1, viewed with a Nikon Optiphot and Plan Apo objectives, and recorded on Pan X using a Kodak No.22 Wratten gelatin filter between field diaphragm and condenser to enhance contrast. For FM and Nomarski optics, fresh specimens were excised from pouches and mounted in half-strength Jensen's medium or water; or in 0.05% aqueous acridine orange (FM
only); for embedded material, LM sections were treated with Na-Ethoxide to dissolve the resin and mounted in 0.05% aqueous aniline blue. Observations were made using a Nikon EF epifluorescence attachment and Nikon Fluor objectives and recorded on Ektachrome 400 daylight diapositive film. Filter sets used were U/420 (365nm main wavelength), V/470 (405nm) and B/515 (495nm).

2.7 POUCH INOCULATION PROCEDURES

For the time course studies presented in Chapters 4-6, the root surface was micro-inoculated. The logic behind this procedure is discussed in Chapter 3. Micro-inoculation involves the placing of a drop of inoculum (approx. 20nl) on a specific region of the root (between the root tip (RT) and smallest emerging root hairs (SERH)). To achieve this, a glass micro-electrode, such as is used for neurological work, was araldited to a 19g hypodermic, which in turn was attached to a 1ml disposable syringe. Micro-electrode inoculation needles (Fig. 2.3) were made from filament borosilicate glass (DA 1.2, DI 0.6) using a David Kopf vertical electrode puller (model no.700c). Plants were pouched as described in 2.5.1 and left to grow for two days until the root tips had reached half way down the pouch. Using a stereo dissecting microscope, the pouch face was cut and peeled back, exposing the roots. A map pin, bent for easy insertion, was put next to the intended micro-inoculation area, pinned into the pouch paper. This prevented the pouch face from interfering with the inoculated area when the pouch face was taped back to its original position. A single or several anion exchange resin beads (Bio-Rad Cl⁻ 100-200um) were placed approximately halfway between the RT and SERH, using fine
forceps. The beads transferred easily (Fig. 2.4) and adhered tenaciously to the root surface, even through fixation and embedding procedures. Control experiments showed that the bead did not affect the root surface or its ability to grow root hairs at that point. Inoculum was transferred to the surface of a single bead using the bead as a guide to droplet size (generally 100um diameter). This procedure demands great care and attention to coordinate not only eye and hand, but also a sufficient small and steady flow of inoculum from the electrode tip. However, with practice the micro-inoculation of one pouch of three plants can be accomplished in less than two minutes, minimizing moisture loss from the exposed roots (Figs. 2.5-2.7).

2.8 TIME COURSE STUDIES

For electron microscopic studies, it is, of course, impossible to study living tissue through infection events, and through the reliability of the micro-inoculation technique to nodulation (and hence infection) time course studies were undertaken involving the fixation of different specimens at different intervals after inoculation. However, one should not lose sight of the fact that by destructive sampling, one loses the opportunity to determine, with reliability, either the sequence of past events or the potential for future events to occur at any given infection site. For these reasons, it was important, when possible, to follow the time course of events at individual infection sites in the living condition, Nomarski optics allowed this to a certain extent, but in some circumstances events, such as penetration of the cell, were impossible to observe using light microscopic levels of observation.
For the time course studies presented in Chapters 4-6, samples were fixed at 2-hourly intervals from 0 hrs to 12 hrs after inoculation; at 4-hourly intervals from 12 hrs to 32 hrs; at 8-hourly intervals from 32 hrs to 72 hrs; and one more sampling at 96 hrs. In all cases, a minimum of ten plants from ten different pouches per sampling period were used in the examination of infection at TEM level. Although it may have been preferable for a larger number of samples, the time needed for TEM work, especially cutting of ultrathin sections and their observation under the transmission electron microscope, precluded larger sampling.

2.9 COMPUTER STUDIES

Images (from thesis micrographs) were fed into an IBAS computer using a 50Hz Newvicon video camera. The processed images were displayed on a Sony Trinitron colour monitor and photographed with Ektachrome 64 slide film. Images were normalized, corrected for shading, and either contour enhanced and mapped, and/or false coloured at close levels of greyness. These studies were performed by Prof. M. Brown, Dr. B.G. Rolfe and Mr. E. Roberts at the Botany Department, University of Texas at Austin, Texas, U.S.A. Such computer processed images have the immediate effect of distinguishing features otherwise difficult to distinguish with the unaided eye. They also have the additional advantage of ease of quantification. For example, when comparing the thickness and densities of cell walls under different conditions (such as infection and non-infection).
CHAPTER 3

ZONE OF INFECTIBILITY IN SIRATRO

The study of susceptibility of the insect vectors to the infectibility of vector-borne infections is a useful means to determine if a vector species is likely to contribute to the transmission of an infection. The discovery of the infectibility of a disease vector, such as a mosquito or a tick, has been a significant step in understanding the ability of the vector to transmit the disease. This chapter will present a similar study for S iratro, because of the importance of the infection localization. The studies presented in this chapter were performed entirely at the laboratory of Dr. Blair Grant, in the University of California, Berkeley, under the guidance of Dr. N. R. H ALL. A list of objectives of studies across the vector-infection infections within the scope of this thesis susceptibility are presented in Chapter 4.

1.7 MATERIALS AND METHODS

1.7.1 BACTERIA

strain AM270 grown in Luria-Bertani medium and stored in 10% glycerol at -80°C. See Chapter 4 for details of this strain.

1.7.2 GROWTH ASSAY

Plants were grown in a greenhouse as described in Chapter 3. Plants were inoculated daily after transfer to bushes, when the plants were 2 leaves on long. A waterproof marking pen was used to indicate the position of the leaf at time of inoculation. The nuts were made very gently to avoid compression of the tissue. After the plant was inoculated, the entire plant was immersed in 20 ml of sterile water and then immediately placed in a laminar flow booth to allow for the natural evaporation of the water. The plants were stored in the booth for 24 hours following the inoculation. This was done to allow for the natural evaporation of the water. The plants were then immediately placed in a laminar flow booth to allow for the natural evaporation of the water. The plants were stored in the booth for 24 hours following the inoculation. This was done to allow for the natural evaporation of the water.
3.1 INTRODUCTION

The study of early events in infection has been hampered by our inability to reliably localise infection to a small region in the root. The discovery by Bhuvaneswari et al. (1980) that soybean has a transient susceptibility to infection, and that this was in a region between the smallest emerging root hairs (SERH) and the root tip (RT), prompted a similar study for siratro, because of the implications for infection localisation. The studies presented in this chapter were performed entirely at the laboratory of Dr. Dietz Bauer, at the Charles F. Kettering Research Laboratories, Yellow Springs, Ohio, U.S.A., under the guidance of T.V. Bhuvaneswari. Results of studies using the micro-inoculation technique within the zone of transient susceptibility are presented in Chapters 4-6.

3.2 MATERIALS AND METHODS

3.2.1 BACTERIA

Strain ANU240 grown to late log phase, cell density approx $10^9$, see Chapter 4 for details of this strain.

3.2.2 PLANT ASSAY

Plants were grown in pouches as described in Chapter 3. Plants were inoculated 1 day after transfer to pouches, when the roots were 4 to 5 cm long. A waterproof marking pen was used to indicate the position of the RT at time of inoculation, the mark was made very gently to avoid compression of the root (see Fig. 2.2). Plants were inoculated by flooding the entire root surface with 200ul of inoculum suspension.
3.2.3 SCORING OF NODULATION

Plants were maintained in the growth chamber for 7 days after inoculation. By this time the most mature nodules on the primary root were usually 0.3 to 1.0mm in diameter and easily scored. Nodules were first visible under the dissecting microscope by 4 days. Small nodules were distinguished from emerging laterals by their spherical (rather than pointed) shape and the browning of infected cells on the top of the nodule. Nodules were counted on a graphics tablet run by an Apple Ile computer using a program written at Dr. Bauer's laboratory. Plants were cut at the RT mark whilst still in the pouch. They were then removed, and using the cut end as the reference point, distances of nodules from RT were tabulated with a digitizing pen. Accuracy was to within 0.1mm.

3.2.4 ROOT ELONGATION

Changes in the position of root epidermal cells, relative to the RT and SERH marks, were assessed by measuring the displacement of ion-exchange beads (Green 1965) placed on the root surface. Growth pouches containing plants with roots 4-5cm long were cut and folded back to expose the roots. Individual beads (Bio-Rad AG1-X8, Cl\(^{-}\), 100-150um) were placed gently with fine forceps on the side of a root at intervals of 0.5mm. The distance of each bead from a fixed point on the pouch was measured to the nearest 0.1mm with the aid of a dissecting microscope. The pouches were resealed with tape and placed in the growth chamber until the next measurement. Measurements were made periodically over a 24 hour period.
3.3 RESULTS

3.3.1 PATTERN OF NODULATION

Nodules on the primary root developed most frequently approximately 2cm. either side of the RT mark (Figs. 3.1-3.3). This is in contrast to soybean where most nodules developed in the region between SERH and RT (Bhuvaneswari et al. 1980). None of the plants developed nodules in the region of the primary root where mature (ie fully-elongated) root hairs were present at the time of inoculation. The location of infectible cells on the root surface at the time of inoculation was deduced from the positions at which nodules subsequently formed on the root, taking the effects of root elongation into consideration (Fig. 3.1B). Root elongation caused portions of the primary root to be displaced relative to the marks made at the time of inoculation on the plastic face of the growth pouch. Ion exchange beads placed on the roots above SERH were not measurably displaced by elongation. Beads initially placed in the zone without root hairs were displaced by root elongation during the first 24 hours of the experiment, but not thereafter (Fig. 3.1B). Roots elongated at the average rate of 1.54 ± 0.25mm/hr (soybean = 2.4 ± 0.6mm/hr). The zone of significant elongation typically extends approximately 30% of the total length of the no-root-hair-zone (NRHZ) (soybean = 5 to 7mm; 50%). Epidermal cells in this lower third of the NRHZ, nearest the tip, were displaced by elongation to positions below the RT mark.

Profiles of nodulation frequency provided further information regarding the location of infectible host cells on the root. The histogram in Fig. 3.1 shows the frequency of nodulation as a function of distance from the RT mark. A maximum frequency of nodulation was observed in the region around the RT mark.
3.3.2 DELAYED INOCULATION

When inoculations were delayed for various intervals, from 2 to 8 hours after marking the positions of the root tips, it was found that nodulation above the RT mark decreased as the time interval between marking and inoculation was increased (Fig. 3.4). The average position of the uppermost nodule decreased in a linear manner in these experiments at a rate quite similar to the average rate of root elongation (14mm in 8 hours, = 1.75mm/hr). The percentage of plants nodulated and mean number of nodules per plant gradually approached and reached zero when inoculations were delayed by 2 to 8 hours (Table 3.1). Average values for the position of the uppermost and for the number of nodules above the mark were quite consistent from point to point within an experiment.

3.4 DISCUSSION

At any given time, the siratro root cells infectible by Rhizobium are located in a small, developmentally-restricted zone just above the root tip. Nodules develop most frequently between 16mm above and 30mm below the RT mark, peaking at RT. Thus, infection occurs within the NRHZ and DRHZ (as in soybean); no infections occurred in the MHZ. At the time of inoculation, the cells in the most-frequently nodulated region of siratro were located in a region 25-50% of the distance between RT and SERH. Since the rapidly-elongating portion of the root typically extends to about 30% of the RT-SERH distance above the root tip, it seems that infections leading to nodulation are normally initiated in epidermal cells that have finished most of their elongation but have not yet reached the stage of root hair emergence.
The data of Cormack (1935, 1945, 1949, 1962) indicate that epidermal cells appear to have a limited capacity for elongation or cell wall formation. This capacity is divided between hair formation and elongation along with axial root growth. If attachment and b layer inhibition by Rhizobium cells occur too late, on well developed hairs, then the cell wall forming capacity of the epidermal cell will be depleted before the tip sliding, hair curling, and infection thread development stages are completed (see section 1.2 and Chapter 4). On the other hand, attachment of rhizobia to epidermal cells that are still rapidly elongating is likely to be ineffectual because there appears to be a fast-acting regulatory mechanism that substantially diminishes the frequency of nodulation in the root zone occupied by these younger epidermal cells at the time of inoculation (Bauer 1982). These considerations lead to fairly precise predictions of the location of the infectible points on the epidermal surface.

Delayed inoculation experiments demonstrated that nodulation above the RT mark decreased rapidly as the interval between the time of marking and time of inoculation was increased. This is consistent with the notion that infections leading to nodule development cannot be initiated in root hair cells that are too old. Hair cells are no longer infectible approximately 6.5 hours (soybean = 5hrs) after they originate from the meristematic zone. Further support for this conclusion is provided by the substantially diminished frequencies of nodulation at distances greater than about 10mm above the RT mark, which is equivalent to about 6.5 hours root growth at 1.54mm/hr. Thus, the transient, acropetal development of infection in siratro should be useful in studies of the infection process.
The average distance of the uppermost nodule from RT mark decreases significantly (to approximately half its initial value) when inoculations are delayed by only 4 hours after marking. It is inferred from this that target epidermal cells of the host root are able to respond to the presence of the bacterial symbiont very soon after inoculation, perhaps within minutes and certainly in less than 4 hours.

The lower frequency of nodulation below the RT mark may reflect the existence of a quick-acting regulatory mechanism in the host that serves to prevent overnodulation.

The Rhizobium normally used for pasture grown siratro is CB756. This strain, a slow-grower, was not used in these infection studies because of its low potential for genetic manipulation compared to the fast-growing ANU240. However, nodulation studies were performed for comparison. Nodulation profiles of CB756 were very similar to those of ANU240, though nodule number per plant was approximately 50% of ANU240 infected plants. The CB756 profile also moved in relation to the root tip when delayed inoculation was performed. It is apparent that CB756 infects at an 'infectibility zone' in a similar manner to ANU240.
## TABLE 3.1

**NODULE COUNT ANALYSIS**

**NODULES COUNTED ON THE WHOLE ROOT**

<table>
<thead>
<tr>
<th>TABLE 3.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>TREATMENT 1</td>
</tr>
<tr>
<td>NUMBER OF PLANTS IN TREATMENT:</td>
</tr>
<tr>
<td>58</td>
</tr>
<tr>
<td>PERCENTAGE OF PLANTS THAT NODULATED:</td>
</tr>
<tr>
<td>12.000</td>
</tr>
<tr>
<td>MEAN NUMBER OF NODULES PER PLANT:</td>
</tr>
<tr>
<td>MEAN NUMBER OF NODULES PER PLANT BASED ON SQUARE ROOT TRANSFORMATIONS:</td>
</tr>
</tbody>
</table>

**CONFIDENCE LIMITS (ERROR BARS AROUND MEAN BASED ON TRANSFORMED VALUES):**

| 90% - NUMBER OF NODULES - UPPER | 12.672 | 12.599 | 11.827 | 11.927 | 11.918 |
| 90% - PERCENTAGE NODULATED - UPPER | 100.000% | 100.000% | 100.000% | 100.000% | 100.000% |
| 90% - PERCENTAGE NODULATED - LOWER | 100.000% | 100.000% | 100.000% | 100.000% | 100.000% |
| 90% - NUMBER OF NODULES - LOWER | 10.000 | 10.000 | 10.000 | 10.000 | 10.000 |
| 90% - PERCENTAGE NODULATED - UPPER | 100.000% | 100.000% | 100.000% | 100.000% | 100.000% |
| 90% - PERCENTAGE NODULATED - LOWER | 100.000% | 100.000% | 100.000% | 100.000% | 100.000% |

**ANALYSIS OF NODULE COUNTS FOR EACH PLANT**

**COMPUTATIONS BASED ON SQUARE ROOT OF NODULE COUNT.**

| THE TREATMENT SUM OF SQUARES: | 2,941 |
| THE ERROR SUM OF SQUARES: | 112,542 |
| THE TOTAL SUM OF SQUARES: | 115,483 |
| THE TREATMENT DEGREES OF FREEDOM: | 4 |
| THE ERROR DEGREES OF FREEDOM: | 257 |
| THE TOTAL DEGREES OF FREEDOM: | 261 |
| THE TREATMENT MEAN SQUARE: | 7.355 |
| THE ERROR MEAN SQUARE: | 0.475 |
| F = RATIO: | 1.546 |
| F = DISTRIBUTION: | 0.1976630635 |

**THE PROBABILITY THAT THE MEANS ARE THE SAME.**

**ANALYSIS OF PERCENTAGE OF PLANTS THAT NODULATED**

| PERCENTAGE OF PLANTS THAT NODULATED: | 100.000% |
| CHI - SQUARE: | 0.000 |
| CHI - SQUARE DISTRIBUTION: | 1 |

**THE PROBABILITY THAT THE PERCENTAGES ARE THE SAME.**
### TABLE 3.1 (CONT.)

**NODULE COUNT ANALYSIS**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Treatment 2</th>
<th>Treatment 3</th>
<th>Treatment 4</th>
<th>Treatment 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>NoteBook Code:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of Plants in Treatment:</td>
<td>50</td>
<td>62</td>
<td>46</td>
<td>44</td>
</tr>
<tr>
<td>Percentage of Plants That Nodulated:</td>
<td>94.8%</td>
<td>92.0%</td>
<td>72.1%</td>
<td>35.3%</td>
</tr>
<tr>
<td>Median Number of Nodules Per Plant:</td>
<td>5.09</td>
<td>3.93</td>
<td>1.59</td>
<td>0.68</td>
</tr>
<tr>
<td>Mean Number of Nodules Per Plant:</td>
<td>5.13</td>
<td>3.89</td>
<td>1.59</td>
<td>0.68</td>
</tr>
<tr>
<td>Mean Number of Nodules Per Plant Based on Square Root Transformations:</td>
<td>4.64</td>
<td>2.57</td>
<td>1.68</td>
<td>1.28</td>
</tr>
</tbody>
</table>

Confidence Limits (Error bars around means based on transformed values):

95% - Number of Nodules - Upper: 5.25 | 2.10 | 5.09 | 1.30 | 0.95 |
95% - Number of Nodules - Lower: 4.01 | 2.17 | 3.93 | 1.13 | 0.78 |
95% - Percentage Nodulated - Upper: 95.6% | 93.2% | 84.5% | 49.0% |
95% - Percentage Nodulated - Lower: 94.3% | 90.9% | 82.1% | 46.9% |
95% - Percentage Nodulated - Upper: 5.34 | 2.08 | 4.54 | 1.41 | 1.04 |
95% - Percentage Nodulated - Lower: 3.76 | 2.12 | 3.99 | 1.34 | 0.93 |

**Analysis of Nodule Counts for Each Plant**

- THE TREATMENT SUM OF SQUARES: 145.194
- THE ERROR SUM OF SQUARES: 93.521
- THE TOTAL SUM OF SQUARES: 240.715
- THE TREATMENT DEGREES OF FREEDOM: 4
- THE ERROR DEGREES OF FREEDOM: 123
- THE TOTAL DEGREES OF FREEDOM: 227
- THE TREATMENT MEAN SQUARE: 36.299
- THE ERROR MEAN SQUARE: 0.403

**F** - RATIO: 90.061

**F** - DISTRIBUTION: 4.471 / 23.15E-12

(The probability that the means are the same.)

**Analysis of Percentage of Plants That Nodulated**

- Percentage of Plants That Nodulated: 62.61%
- Chi-Square: 128.325
- Chi-Square Distribution: 3.104 / 0.07E-09

(The probability that the percentages are the same.)
INFECTION OF SIRATRO BY STRAIN ANU240

1. ATTACHMENT AND CURLING

Despite the intense interest in the field, there is a lack of published results about infection structure at the level of the electron microscope, reflecting the complexity of bacterial-plant associations and the need for further extensive analysis.

In these next three Chapters, the presented micrographs represent the major events observed in the process of infection of siratro, and have been selected from a large amount of material processed and observed during these studies. A summary of these events is presented in Fig. 4-1.

A. ATTACHMENT OF RHIZOPLAS TO ROOT HAIRS: A MOST SPECIFIC INTERACTION

It is worth mentioning at the outset that specificity between Rhizobium and legumes is likely to find expression at any of the following levels of interaction between the symbionts:

a) Colonization of the root surface.

Rhizobia, like other rhizosphere-attached soil bacteria, are more numerous in the vicinity of plant roots than in the soil away from roots (Daré & Sanders, 1961). They are likely to be more stimulated by legumes than by grasses. Any specificity at this stage is likely to be of low order.

b) Nod-factor response.
4.1 INTRODUCTION

This Chapter, and Chapters 5 and 6, combine results and discussion sections, making it easier to introduce ideas and hypotheses relating to the results.

Despite the intense interest in the field, there is a lack of published results about infection structure at the level of the electron microscope, reflecting the complexity of bacterial-plant associations and the need for further extensive analysis.

In these next three Chapters, the presented micrographs represent the major events observed in the process of infection in siratro, and have been selected from a large amount of material processed and observed during these studies. A summary of these events is presented in Fig. 4.1.

4.1.1 ATTACHMENT OF RHIZOBIUM TO ROOT HAIRS - HOST SPECIFIC INTERACTIONS

It is worth mentioning at the outset that specificity between rhizobia and legumes is likely to find expression at any of the following levels of interaction between the symbionts:

a) Colonization of the root surface.

Rhizobia, like other rhizosphere-stimulated soil bacteria, are more numerous in the vicinity of plant roots than in the soil away from roots (Dart & Mercer 1964). They are likely to be more stimulated by legumes than by grasses. Any specificity at this stage is likely to be of low order.

b) Root-hair response.
Earlier reports give the impression that curling is a relatively non-specific effect, but if the nature and frequency of root-hair deformation is taken into account it becomes apparent that it is almost as specific as nodulation itself (Haack 1964; Yao & Vincent 1969). Whatever the process of initial invasion it is certainly very specific, and affected by the genetic constitution of both partners. Hosts range widely in their susceptibility to invasion. There is also a degree of specificity which determines the number of root hairs invaded and the proportion of these invasions which results in nodules (Nutman 1959).

c) Invasion and formation of an infection thread, formation and persistence of a nodule meristem.

d) Intracellular release of the invading rhizobia, multiplication of rhizobia within the membrane envelopes and conversion to the bacteroids.

e) Establishment and maintenance of an efficient, integrated and shared metabolism between the macrosymbiont and the microsymbiont (Vincent, 1974).

4.1.2 TIP GROWTH

To appreciate fully the mechanism of curling after attachment of_ Rhizobium, it is first necessary to understand tip growth. A model of root hair tip growth based on Jaffe (1982) is presented in Fig. 4.2. In normal tip growth, vesicles containing wall material move to the dome tip and fuse with the wall, contributing new wall substances and hence tip growth (see Chapter 7). Jaffe has proposed that the tip is a site of ion leakage which allows calcium to enter the tip. The calcium is immobilized there (possibly by chelating agents) and its
positive charge draws the vesicles, by self-electrophoresis, to the tip. These vesicles would contain not only new wall substances to generate new wall volume, but also new ion pores. This view of tip growth as a self-sustaining loop process is a major conceptual advance (Green & Poethig 1982). In the model, the hair tip is presented as an hemispherical dome, a simplification for ease of explanation. A more appropriate outline would be semi-elliptical. In either case, it is important only to understand that tip growing cells such as root hairs (and pollen tubes and fungal hyphae) extend their cylindrical form by maintaining a specific gradient in the rate at which area expands within the growth zone; this rate falls, generally as a cosine or cotangent function, to zero at the base of the zone (Green & King 1966). Thus, any inert object attached at the dome tip will be quickly moved away towards the point of zero growth at the dome equator. The biophysical basis for this gradient and the cessation of growth at the base of the dome is not obvious. The stresses tending to enlarge the wall are actually greater there than at the tip. The cell wall cross-section shows no increase in thickness or density in the electron microscope, which could explain the halt of growth (Green 1963). One suggestion is that the gradient, and the ultimate cessation of growth, results from two contrasting properties of the carbohydrate-containing vesicles which fuse with the wall to enlarge it (Green 1973). The vesicles would fuse preferentially at rapidly expanding regions. Upon arrival the carbohydrate would 'loosen' the wall, promoting extension and hence the fusion of still more vesicles. This positive feedback activity enhancing growth would be checked generally by an upper limit on the rate of vesicle production and locally by the strain (deformation) hardening properties of the
carbohydrate once it is in the wall. The cumulative stiffening effect would increase with distance from the tip to bring on stoppage at the base of the dome.

4.1.3 ATTACHMENT

The very nature of curling suggests that Rhizobium attachment to the hair tip is an active event. It is proposed that a bacteria attaches at or near to the growing apex of the hair and is quickly moved away towards the dome equator. However, because of the active processes by Rhizobium it never reaches the point of zero growth (modeled in Fig. 4.3). Thus, Rhizobium presumably perturbs the loop process of tip growth below its attachment site. This is the single most important event in the initiation of curling. The exact nature of attachment, and the rapidity with which Rhizobium can inhibit tip growth processes beneath it, will decide the future of the hair, i.e. if it will curl or not. This inhibition, and the rapidity with which it is carried out, may thus be a very early deciding feature of specificity. If the specific interaction between tip and Rhizobium is inexact and the action of the bacteria is slowed, then it will reach the dome equator and there will be no curling. As attachment occurs generally across the plant spectrum, regardless of host specificity, it is suggested that the ability to inhibit tip growth processes is the first specific infection step, and hence the first deciding factor of host specificity. Thus, if the inhibition factor is a simple hydrolytic enzyme (see Chapter 5), then this first possible major host specificity step is likely to be controlled by a single Rhizobium gene.
4.2 MATERIALS AND METHODS

4.2.1 BACTERIAL STRAIN ANU240

Strain ANU240 is a streptomycin-resistant derivative of NGR234. Of over two hundred isolations made in New Guinea from nodules of the cowpea group of plants (which are normally nodulated by slow-growing rhizobia, the Bradyrhizobium), Trinick (1980) found only one fast-growing Rhizobium. This strain, NGR234 (isolated from Lablab sp.) resembles fast-growing rhizobia from the tropical tree Leucaena leucocephala, and is able to nodulate a broad range of the cowpea group of plants, as well as lucerne (Medicago sativa), Leucaena and Acacia farnesiana (ibid). In addition, this fast-growing Rhizobium is able to nodulate the non-legume Parasponia (Trinick and Galbraith 1980). In contrast to the Bradyrhizobium from Parasponia, the symbiosis between Parasponia and strain NGR234 is ineffective in nitrogen fixation.

4.2.2 PLANT ASSAY

This study was undertaken using a time-course fixation schedule using micro-inoculation procedures on pouch grown plants (see Chapter 2).

4.2.3 MICROSCOPY

Specimens were fixed, embedded and observed according to schedules set out in Chapter 2. Where possible, fresh material was observed continuously by Nomarski optics.
4.3 RESULTS AND DISCUSSION

4.3.1 ATTACHMENT

Strain ANU240 attaches to the root surface within minutes of inoculation (see also Turgeon & Bauer, 1982). This attachment occurs generally over the root surface and is often polar (Figs. 4.4-4.7). However, observations show specific attachment at root hair (RH) initiation sites and at emerging RH tips (Figs. 4.4, 4.5).

4.3.2 CURLING

TEM observations of Rhizobium effects on the surface of cell walls show that the Rhizobium interferes with the way the microfibrils are laid down. The wall microfibrils at the pivot of the curl are aligned at 90° to the plane of the hair wall, but are parallel elsewhere (see Figs. 5.19, 5.20, 11.14 and 11.15). In other examples, the orientation of fibrils below a microcolony on the surface of a hair are 90° below the colony but parallel to the plane of the hair surface elsewhere. These observations prompted the model presented in Fig. 4.38. It is assumed that microfibrils are normally laid down parallel to the plane of the wall and polymerise into long cellulose chains under the direction of the underlying components of the cytoskeleton (Edelman 1976). However, if the Rhizobium secretes hydrolytic enzymes that inhibit the polymerization process, and if there exists a vertical gradient through the wall, such as may be created by a flux of calcium creating a pH or electric gradient, then the microfibrils will align at 90° to the plane of the cell wall (Fig. 4.3B). Under these conditions, the resulting changed wall area can readily be seen to be far more flexible than the rest of the cell, and
it is here that the tip pivots over to form the curl. These ideas are especially attractive in view of the recent results of Goosen-de Roo et al. (1984) who describe the b layer of the cell as developing on the outside of the hair cell in pea (see Chapter 1). The result is modeled in Fig. 4.11, where a series of hemispheres represents stages in curling. This model was drawn directly from micrographs of curled hairs (Figs. 4.21, 4.23 and 4.24). Thus, curling, which results from one-sided tip growth inhibition by Rhizobium, can result in hair curling only when three conditions are simultaneously fulfilled:

1. Root hair surface growth beneath the attached Rhizobium is inhibited,
2. Rhizobium growth inhibition is limited to one side of the hair,
3. Rhizobium growth inhibition is rapid enough and effective enough to prevent removal out of tip growth range

The nature of curling suggests that the area of greatest growth at the tip is larger than a single or several bacteria can cover or affect enzymically at a single moment.

Why is curling such a necessary part of infection when Rhizobium can affect cell walls at various parts of the root surface?

Penetration by the bacteria (Chapter 5) does not occur at the pivot of the curl, as previously suspected (Napoli & Hubbell 1976; Callaham & Torrey 1981), but in a region between the pivot and the root hair tip. In addition the penetration does not involve reorientation of the wall microfibrils but the dissolution of the mature wall matrix and depolymerization of the microfibrils (as opposed to prevention of polymerization in hair curling). Thus, reorientation effects by
Rhizobium on plant cell walls could be the expression of Rhizobium 'hair curling genes' (Hac), and degradation and penetration of the hair cell could be the expression of genes such as for hydrolytic enzymes, these could be called 'penetration genes'. Effects, such as those that may be caused by the action of such genes can be seen in Figs. 4.7 - 4.10 and 4.12 - 4.16. It will be important to know what kind of environmental conditions are switching these genes on and off. Presumably the products of penetration genes are not successful beneath any ordinary colony at the surface, and undoubtedly need to be enclosed in the small pocket of the curl in order to achieve one or a combination of the following:

1. appropriate concentration levels of these gene products;
2. the avoidance of high concentrations of other, inhibiting substances, much as nitrogenase needs low oxygen tension;
3. to increase the concentration of signals and effectors from the microsymbiont to the host, or host to microsymbiont, that would otherwise leak away laterally from the site;
4. increased concentration of nutrients for the microsymbiont.

The action of bacterial enzymes appears to be random, as seen in the occurrence of holes in the surface mucilage of the root (Figs. 4.12-4.16). This does not occur with the nodulation-defective mutant strains ANU265 and ANU1255 (Chapter 9), nor (of course) in minus-Rhizobium controls (Chapter 7).

The hair cell doesn't burst from release of turgor due to the penetration of the bacteria, nor is there apparent bulging of the plasma membrane outwards through the point of bacterial entry. It is
therefore assumed that either the curl forms an effective seal against pressure, or the bacterial polysaccharide exudates act as a sealing agent, or both (see Ljunggren, 1969).

4.3.3 TIMING OF CURLING EVENTS

By 5-6 hours after inoculation the hair begins to grow asymmetrically due to the one-sided, 'pinning-down' effect of the bacteria (Figs. 4.17-4.20).

Between 5 and 11 hours the hair gradually curls and contacts its own basal part or neighbouring cell and usually continues to grow along the epidermal surface (Figs. 4.11, 4.13, 4.20 and 4.23). Presumably at this moment normal tip growth resumes before it is 'delegated' for infection thread synthesis (see Dart 1977). Infected hairs sometimes continue to curl in a corkscrew manner (Fig. 4.12). This would appear to occur when the hair pivots at an angle smaller than 90° and misses the epidermal surface, continuing to circle.

By 12 hours, curling is complete. In Fig. 4.21 a curled hair, split longitudinally, has lost its cytoplasmic contents in the process of SEM preparation, revealing the bacterially affected site. This figure demonstrates unequivocally that Rhizobium not only acts almost immediately on attachment, judging by the position of the pivot of the curling axis, but also attaches when the hair is just emerging from the trichoblast (compare Figs. 4.21 and 4.22, 4.11 and 4.3). This is also important in light of the results presented in Chapter 8, in which is described the action of the lectin RCA 1, which is a specific probe for the sugar β-D-Galactose, that is able to detect sites of emergence of root hairs before they can be observed by any other LM method. There is no reason why Rhizobium is not also able to 'detect'
this or another compound (such as phosphorylase - see Chapter 7) before their emergence and attach to this site accordingly. Analysis of curled hairs by normal hair growth rates (6.0\text{um hr}^{-1}0.5\text{um}) suggests that the \textit{Rhizobium} attaches to the root hair tip within the first 1.5 hours after inoculation.

The hair appears to maintain a helicoidal arrangement of the hair wall microfibrils (see Pluymaekers, 1982). The nucleus is always observed next to the future site of penetration (Fig. 4.23 and 4.24)(see also Figs. 5.3, 5.10 and 5.14) and, as demonstrated by Nutman \textit{et al.} (1973), the proximity of the nucleus is of crucial importance to the entire infection process.

4.3.4 BROWNING OF INFECTED HAIRS

About 12 hours after inoculation a brown colouration of the infected hair wall becomes apparent. This browning affects the whole trichoblast cell at the root surface, and may be the beginning of the wall 'thickening' observed in LM and TEM (see Figs. 5.19, 5.23 and 6.11), and is reminiscent of other host-pathogen interactions (Callow 1977). It was initially thought that this thickening was the uncontrolled deposition of infection thread material, whereby the mechanism for infection thread synthesis had been 'primed' by curling but was as yet undirected, because penetration does not occur until about 22h after inoculation. However, though young infection threads and the inner part of the infected wall have similar grey values (see Fig. 5.22), infection threads stain a distinct purple (cellulose) with toluidine blue, whereas the infected wall stains sea-green, similar to lignin. It may be possible that the mechanism for this deposition is similar, but the deposition substances are different. The curled
hairs appear as small brown 'lesions' when viewed under the light microscope, and can be easily distinguished with fluorescence microscopy (Fig. 4.25). It is possible that the browning represents deposition of phenolic compounds. Another possibility - callose deposition - was eliminated by testing for fluorescence after staining with aniline blue. The result was negative and control (slime plugs in phloem sieves) positive.

On flood inoculation, curling can occur on a massive scale, not only in the transient zone (Fig. 4.26) but also amongst the mature hairs (Fig. 4.27). Note how different the curling is in the transient zone, where curled hairs are always very close to the surface, making observation by light microscopy very difficult indeed. Because of the occurrence of root hair curling over the whole root, infection of siratro at the transient zone of infectibility may not be controlled by 'curling genes', even though they are highly specific. Instead, it is possible that infectibility and subsequent nodule occurrence at the no-root-hair-zone may be a function of pericyclic readiness (see Chapter 6).
CHAPTER 5

INFECTION OF SIRATRO BY STRAIN ANU240

II. PENETRATION AND INFECTION THREAD SYNTHESIS

The following sections attempt to answer some of these questions. Please refer to Fig. 4.1 throughout this chapter.

The major sections of the book are numbered.

5.1.1 CELL WALL DEGRADING ENZYMES

Plant cell walls are composed of highly complex arrangements of protein and carbohydrate-containing polymers (Wise et al., 1981).

Plant cell wall growth and deposition is dependent on synthesis and degradation of complex polymers. The penetration of plant pathogens frequently requires the production of enzymes that mediate the
5.1 INTRODUCTION

There are many unanswered questions regarding penetration and infection thread synthesis in the infection process. For example,
1. How does the thread grow inward against host turgor pressure?
2. What causes the tip of the growing infection thread to follow the hair cell nucleus?
3. How does the infection thread penetrate into adjacent cortical cells?
4. How are cortical cell divisions stimulated in advance of infection thread penetration?
5. How are the normal defence responses of the host to microorganisms avoided or suppressed by the invading rhizobia?
6. When does information transfer between Rhizobium and legume start and in what form?

The following sections attempt to answer some of these questions. Please refer to Fig. 4.1 throughout this chapter.

The Figure 5.3 consists of LM micrographs to which the TEM micrographs in Figures 5.4-5.10 are relevant. Details are discussed in the Figure legends.

5.1.1 CELL WALL DEGRADING ENZYMES

Root cell walls are composed of highly complex arrangements of pectin and carbohydrate-containing polymers (McNeil et al. 1984).

Plant cell wall growth and deposition is dependent on synthesis and degradation of complex polymers. The penetration of plant pathogens frequently involves the production of enzymes (ie cellulolytic and
pectolytic) that degrade the host cell wall. *Rhizobium* penetration into root hair cells appears to be mediated by the action of pectolytic and cellulolytic enzymes. Lunjggren & Fahraeus (1959, 1961) suggested that infection occurs via the release of pectin degrading enzymes that loosen the wall, and that cell wall degrading enzymes are released by the plant in response to specific bacterial polysaccharides which pass through the cell wall and interact with the the host plant nucleus, thus stimulating enzyme production. They also suggest that homologous strains appear to induce more pectolytic activity than do heterologous strains, thus resulting in cross-inoculation specificity. Verma et al. (1978) reported soybean infection by *Rhizobium* involved both pectinase and cellulase. Pectinase activity was associated with the bacteroids, whereas cellulase was localised in the cell wall region of the infection thread. Pectinmethylesterase was found in both inoculated and uninoculated root exudates.

The endopectic enzymes, which cleave the α-1,4 galacturonic bonds in the rhamnogalacturonic fraction of the cell wall, represent the key to enzymatic decomposition of cell walls in herbaceous tissues (Basham & Bateman 1975ab).

The pectic enzymes possess the potential of playing a dual role in pathogenesis (cell death and tissue maceration by dissolution of the middle lamella and weakening of the primary wall structure by solubilization of the pectic fraction) in addition to rendering the non-pectic polymers in cell walls more accessible to enzymatic hydrolysis (Bateman & Basham 1976; Bateman 1976).

It is apparent that the problems of cell wall degradation during legume infection and in pathogenesis are complex. A number of factors
may determine whether or not a particular enzyme is produced by a microsymbiont or pathogen in a given environment. If the enzyme in question is produced, the environment into which it is excreted can be expected to greatly influence its effectiveness and/or its existence in an active form. The molecular environment at the host-bacterial interface and in invaded tissues is likely to contain elements that influence enzyme induction or repression in the bacteria as well as activity and stability of the different wall degrading enzymes (Albersheim & Anderson 1971). Furthermore, the problems of substrate accessibility become relevant since certain wall polymers may be masked by others and deposition of lignin-like materials in cell walls during infection represents a common phenomenon (Mullen & Bateman 1975) that may also protect cell wall polysaccharides from enzymatic hydrolysis.

Ljunggren and Fahraeus (1961) indicated that infection of legumes was strongly correlated with the production of polygalacturonase (PGA). They concluded that PGA plays an important part in the infection process. This function was thought to be a weakening of the cell wall of the root hair, facilitating bacterial invasion. The formation of PGA was thought to be the primary effect, resulting in a partial depolymerization of the cell wall pectin, facilitating bacterial invasion. PGA is always present in the growing root hair in small amounts: the apical hair wall consists mainly of pectic substances, and the function of PGA may be a continuous softening of this wall, which leads to cell elongation (Preece 1982). However, when appropriate Rhizobium are present, they may induce, at least in some hairs, a much stronger production of polygalacturonase. This might result in a more pronounced depolymerization of the pectic layer, allowing the bacteria to penetrate the cell.
Unfortunately, others have been unable to find pectolytic activity for *Glycine max*, *Trifolium repens*, or *Medicago sativa* under similar experimental conditions to those performed by Ljunggren and Fahraeus (Solheim & Raa 1971, Banish 1973b); Solheim & Raa found significant activity associated only with fungal contaminants. Banish concluded that the cell-wall degrading enzymes are present during seedling growth at activities that reflect the changing growth conditions rather than bacterial effects.

It is apparent from recent studies of the influence of pectic enzymes on plant tissues, that if cell wall degrading enzymes are involved in establishment of obligate parasites, which have or require intimate contact with living host cells, the production of cell wall degrading enzymes by such parasites must be subject to precise regulation and control, to avoid killing the host cells (Basham & Bateman 1975ab). Further elucidation of the systems regulating production and excretion of cell wall degrading enzymes by plant pathogens should add considerably to our understanding of parasite-host and *Rhizobium*-legume relationships.

5.1.2 INFECTION THREADS

Infection threads are appositional wall growths and are probably analogous to wound plugs and papillae. Papillae are localised appositional wall structures that frequently form during penetration of fungal pathogens (Aist 1976; Vance et al. 1980). Papillae have been implicated in disease resistance in many plant pathogenic fungus-host plant interactions. Successful infections occur presumably because the pathogen can breach the wall more rapidly than the completed apposition can form (although Aist (1983) reports that
papillae can form in seconds). Aborted infection threads may represent a successful resistance response against Rhizobium infection. The tip of the infection thread may become walled off, preventing contact and recognition between the plant and bacterium. In contrast, continued growth of the infection thread may reflect non-closure of the tip and continued successive deposition of wall material (Newcomb 1981).

In many respects, infection thread synthesis could be a repair mechanism, where signals for repair overpower signals for tip growth, and resources are re-allocated. It can thus be seen as the 'continuous repair of a continuous irritation'. This is a very important step. Until now the process of infection could be seen as a pathogen-host incompatible response (Dickinson & Lucas, 1982; see also Vance, 1983), bacteria multiplying in an 'intercellular space' (the curled hair enclosure), and causing disintegration of the host cell wall. However, instead of destroying the host tissue, the bacteria 'communicates' with the host, possibly through a plasmalemma of altered permeability (Wheeler, 1976), and infection thread synthesis ensues.

Histochemically, the thread wall contains the same components of pectic substances, hemi-celluloses and some cellulose, as the root hair tip, and this is supported by electron micrographs of infection threads in root hairs and nodules (Sahlman and Fahraeus 1962; Dart and Mercer 1963; Higashi 1966). The thread has a certain rigidity. Thread growth thus results from plant cell wall deposition to confine a growing colony of rhizobia which in turn prevent the plant cell wall enclosing them in a structure rigid at its tip (Newcomb 1981). Aborted
infection threads may be those where Rhizobium growth was too slow to prevent the thread wall becoming completely rigid.

The direction of the thread growth and its close relation to the nucleus might reflect a nuclear influence on the direction of cytoplasmic streaming which then directs the deposition of wall material into the characteristic tube structure. The thread in the curled part of the root hair is often itself curled and may branch and anastamose, possibly again reflecting the changing directions of cytoplasmic streaming.

Rhizobia have the ability to induce breaks in plant cell walls, because the infection thread crosses already-formed walls on its progress through the cortex, developing as a tube joined at each cell junction to the wall of the cell it is crossing. The cortical cell wall 'dissolves' and joins the infection thread. A characteristic swelling of the intercellular space and often large accumulation of rhizobia occurs before the thread enters the adjacent cell. As in the root hair, the infection thread and nucleus are closely associated. The infection thread may branch many times, often at intercellular spaces (see Dart 1977; Newcomb 1981). Ultimately within the nodule a gap develops in the thread wall to release the rhizobia, often in a vesicle formed as a swelling of the thread tip.

Rhizobium is presumed to produce the 'zoogloeal matrix' surrounding the bacteria inside the more rigid wall of the infection thread. This matrix stains with the basophilic dye toluidine blue in similar fashion to the RNA of the host nucleus, and also with Schiff's reagent and Alcian blue, suggesting the presence of mucopolysaccharides (Dart 1977).
Not all infections give rise to nodules (Ward 1887). Many infections abort before reaching the base of the hair, others abort at the base of the hair and in the root cortex (Nutman 1959; Calvert et al. 1984). The nucleus associated with aborted threads often rounds up, becomes smaller and appears to degenerate (Fahraeus 1957).

5.2 MATERIALS AND METHODS

See section 4.2

5.3 RESULTS AND DISCUSSION

5.3.1 MICROCOLONY GROWTH

Between 12 and 20 hours *Rhizobium* colonizes the pocket between the curled hair and the epidermal surface. Transverse sections through a curled hair (curling in a plane vertical to the paper) show that the bacteria extensively colonize this space (Figs. 5.1, 5.2). Such colonization was observed in all 20 samples observed by TEM. However, no attempt was made to gauge the frequency of these colonized spaces on a root surface area basis. It is clear that any enzymes necessary for degradative penetration of the hair cell could be concentrated in this small area.

5.3.2 PENETRATION EVENTS

Penetration occurs 20-22h after inoculation. Two types of penetration of the hair have been observed: either at a single point or a small zone.

5.3.2.1 Penetration at a single point
Penetration of a hair at a single point (Fig. 5.5) is similar to penetration of wheat epidermal and cortical cells (Foster et al. 1983). In early stages of pathogenesis it is known that marked structural modifications occur in host cell walls. These arise as small blister-like protrusions containing densely-staining amorphous or membraneous material embedded in an electron-lucent matrix, continuous with the host cell wall (Luke et al., 1966; Hanchey et al., 1968). Similar cell wall modifications, variously termed lomasomes or lomasome-like (Ehrlich & Ehrlich, 1971), paramural bodies (Israel & Ross, 1967) or boundary formations (Tu & Hiruki, 1971), have been found in many diseased plants (see also Wheeler, 1976). In Fig. 5.5 the host cytoplasm has moved away from its wall, leaving an 'interfacial' zone, possibly of soluble pectins, and possibly containing lomasomes, plasmalemmasomes and paramural bodies.

5.3.2.2 Penetration at a small zone

This second type of penetration has similar cytological appearances. Fig. 5.11 shows a section through an infection site, the small zone between the curled hair and epidermis. The microcolony of rhizobia appears to have degraded the cell wall matrix, leaving a layer of 'depolymerized' wall microfibrils (Fig. 5.12). A single Rhizobium was presumably in the process of penetrating this loosened wall material at the time of fixation, possibly as a result of colony growth pressure. The host cytoplasm has moved away from its wall, leaving an interfacial zone, where accumulation of plasmalemma-like vesicular bodies may represent the first deposits by the host of infection thread-forming material. These vesicles may also contain molecules which are important in interactions with the invader (see Ingram et al. 1976). This interfacial zone may in fact be the result of
plasmolysis, and other workers have had similar problems (P.J.Dart, pers. comm.). Recent information (S.Tiwari, pers. comm.) implies the use of phosphate buffers, and now PIPES buffer is recommended. The hair cell cytoplasm is dense and granular, and microtubules are present next to the infection site. Microtubules may play a role in the direction of infection thread wall material synthesis (Figs. 5.8 and 5.9). It is also possible that the underlying components of the cytoskeletal system are connected to at least some of the receptors involved in recognition events during penetration.

In contrast to previous results (Napoli & Hubbell 1975; Callaham and Torrey 1981), the infection is not directly at the axis of the curl, the rhizobia entering into the hair cell in an area between the axis of the curl and the hair tip.

These observations demonstrate that a region of the host cell wall is degraded by rhizobia, and it seems likely that the cell wall is altered by hydrolytic enzymes, either from the host and/or from the rhizobia. The polygalacturonase hypothesis (Ljunggren and Fahraeus 1959; Bauer 1981) is fully compatible with the evidence presented here: rhizobia multiply in an enclosed pocket and locally disrupt or degrade the hair wall, and a new layer of host cell wall-like material is deposited at the infection site. Whether the accumulation of this new wall-like material is a defence response is by no means proven, but is tenable. Certainly, the localized deposition of wall-like materials such as papillae, collars, or sheaths at the point of penetration of microbial pathogens is well known (Fullerton 1970; Bracker and Littlefield 1973; Aist 1976; Aist & Israel 1977ab; Sequeira et al. 1977). For example, at the site of attachment of avirulent and incompatible Pseudomonas solanacearum to tobacco
mesophyll cells (Sequeira et al. 1977), the host cell wall is frequently eroded, the plasmalemma separates from the cell wall and becomes convoluted, and numerous membrane-bound vesicles accumulate in the space between plasmalemma and the cell wall (the interfacial zone). A hypersensitive response develops and, as a result, the host cell collapses and organelles are deranged. Virulent strains do not attach and cause no visible alteration of plant structure. Recent reports indicate that fragments of pectic polysaccharides from the host cell wall can act as potent elicitors of defence responses in plants (Lyon and Albersheim 1980; Hahn et al. 1980). Thus, localized activation of host root polygalacturonase might result in both localized degradation of the hair cell wall and localized release of pectic elicitor substances that induce deposition of infection thread material (Bauer 1981).

5.3.3 DOUBLE MEMBRANE STRUCTURES

Double-membrane structures were observed within the microcolony, but not within the host wall microfibrils or interfacial zone (Fig. 5.12). It is tempting to hypothesize that these are host- and/or rhizobial-secreted fragments, bound with proteins or glycoproteins, that function by signaling the initiation of the complex reactions leading to the symbiosis.

From work on the isoagglutinins of Chlamydomonas (see Heslop-Harrison 1978), it is now known that these very high molecular weight substances (in the region of $1 \times 10^8$ daltons) are not molecules but pieces of membrane. Viewed with the electron microscope after separation by density gradient centrifugation they are seen to be vesicles with fuzzy coats, and the coats may well be sites of the
factors concerned with recognition and adhesion. These vesicles are derived from the membranes of the flagella, and it seems likely that they are shed continuously at the tip, the membrane being replenished by the flow of new membrane substance from the base. The factors concerned in recognition and adhesion are presumably held on the outer surface of the membrane, and the configuration of the surface proteins and the nature and distribution of the carbohydrate portions of glycoproteins presented to the environment at any one time would depend on the metabolic activities of the cell during the synthesis of the particular bit of membrane currently exposed, itself determined by which genes happened to be available for transcription. It is interesting that coated vesicles (as well as smooth vesicles) have been observed in the infected nodule cell (Robertson et al. 1984a) and are thought to be involved in the biogenesis of infection thread membranes and possibly infection thread matrix material and peribacteroid membrane. It is also possible that such vesicles are carriers of 'message molecules' involved in recognition, which may explain why some kinds of ineffective mutants (Chapter 12) disfunction at the stage of bacterial release.

5.3.4 INTERFACIAL ZONE AND PLASMALEMMMA

During the establishment of a compatible, intracellular relationship between a biotroph and its host the plasmalemma of the penetrated cell or cells is invaginated to accommodate the parasite. At the same time there occurs a complete reorganization of the metabolism of the infected cell, and sometimes adjacent cells, to meet the needs of the invader (Brian 1967; Scott 1972). Such changes may, in some instances, result from the mere physical presence of the parasite (eg
as a non-specific wound response resulting in the deposition of callose) or, in the majority of cases, as an alteration in the expression of the host's genome in response to regulatory molecules secreted by the parasite, or attached to its surfaces. At the structural level, alterations in metabolism may be reflected in a number of ways, both immediately before and during penetration. Although the work of Williams et al. (1973) on the infection of *Brassica* (where he showed that the infection of the root hair of *Plasmodium brassicae* occurs by injection of the parasite through a puncture in the hair wall) throw some light on the nature of the relations between host and parasite, further progress will only come from investigation of the interface between the two organisms, and cytochemical and cytological studies of interaction during the first few moments of the relationship. Recognizing that the interfacial membranes occupy a key position during the establishment of a compatible relationship between *P. brassicae* and its host, Aist (1974) investigated their origin and structure using the techniques of freeze-etch microscopy. Besides revealing the complex nature of the interfacial membrane region, he was able to show that the development of the outer plasmodial envelope, which initially derives from the invaginated plasmalemma of the host, is partially directed by the parasite. It seems reasonable to speculate that the appearance of this altered membrane is a prerequisite for proper exchange of materials between host and parasite. Studies of interfacial membranes are in great need of further sophisticated biochemical and cytological investigation to uncover the functional significance of the specializations which occur.
5.3.5 INFECTION THREAD SYNTHESIS IN SIRATRO

By 24h after inoculation, root hair tip growth ceases and is redirected to infection thread synthesis (Dart, 1977) as bacterial division causes 'pressure' on the plasmalemma. The thread matrix is similar to the interfacial zone and is interconnected. The nucleus is very close to the thread-forming site (Fig. 5.14A), and may be involved in thread synthesis. Threads are formed by extensive vesicular fusion (Fig. 5.14B,C) (see also Newcomb 1981) and ramify throughout the hair (Figs. 5.15 and 5.16). They do not orient towards the cortex until approximately 40 hours after inoculation (Fig. 5.18). Brown colouration of the infected hair is now more easily seen (see Fig. 6.11). An example of two infection sites in a single hair demonstrates the extensive ramification of threads that occurs in infected hairs (Fig. 5.16). Serial sections were made through the entire sample for clear proof of a double infection (which most likely occurs when attachment of a Rhizobium is followed by another within a short period of time). The hair wall lifts slightly at the penetration zone (perhaps as a remnant of the bacterial colony pressure) and joins appositional wall material deposited during penetration (Fig. 5.16C, 11.13 and 11.14), see Callaham and Torrey (1981) for a very clear description of this phenomenon.

5.3.6 PENETRATION OF THREADS INTO THE CORTEX

By 40-52 hours after inoculation, threads orient towards the cortical tissue, penetrating and ramifying through the focus (or meristem) of cells. They are easily observed with all types of microscopy used (Figs. 5.18-5.20, 5.23 and 5.24).
By 52 hours, threads are 'delivering' bacteria into the still-dividing tissue. The bacteria divide and fill the host cells, swelling into bacteroids. Tests for any callose involvement using aniline blue staining and FM were negative.

There appears to be differences in the cell wall characteristics between young and mature infection thread walls. There are also differences between the outer (thickened) wall of the infected hair and the basal wall of the same cell. See Figs. 5.21 and 5.22 for details.

Aborted infections can usually be found close to growing nodules in the infectibility zone. They can be distinguished by an accumulation of phenolic-like substances (Fig. 5.25A) and/or autofluorescence of the infection thread (Figs. 5.25BC and 5.26), a phenomenon that was not seen in successful infections. Aborted infections were not studied in detail.

5.3.7 A MODEL OF PENETRATION AND INFECTION THREAD INITIATION

A model summarizing the results presented in this Chapter is given in Fig. 5.27. See the Figure legend for details. From the Figure, it is clear that the main events of penetration and commencement of infection thread synthesis are in great need of further and intensive study. The overall objectives of this thesis did not allow sufficient study that these events deserve.
CHAPTER 6

INFECTION OF SIRATRO BY STRAIN ANU240

III. CELL DIVISION

...
6.1 INTRODUCTION

One of the earliest signs of successful infection when viewed by the unassisted eye or by dissecting microscope is the small surface swelling caused by the cell division of the forming nodule. At this stage it is easy to mistake emerging lateral roots for nodules, because both cause epidermal tearing as they push through the epidermal tissue. However, if the same site is observed periodically over a few hours, the sharp-ended form of the lateral root is easily distinguished from the round form of the emerging nodule. This Chapter describes the timing of the first cell divisions stimulated by infection.

Early cell division in siratro infection was not studied in great detail for this thesis, because of work in recent publications (eg Newcomb 1981; Turgeon & Bauer 1981). However, early detection of cell division is important to distinguish certain kinds of mutant Rhizobium deficient in stimulating or maintaining stimulation of plant cell division.

6.2 MATERIALS AND METHODS

For Computer studies and microscopic methods refer to Chapter 2.

6.3 RESULTS AND DISCUSSION

Cortical cell division does not start until approximately 22 hours after inoculation. The first indication is an enlargement of the subepidermal cell nucleus and nucleolus below the infection site (Figs.
The use of acridine orange staining, enhanced by computer imaging, can detect nucleoli enlargement at the earliest stages. Such samples, viewed under TEM, show cells ready for division (Figs. 6.4, 6.5). By 48 hrs after inoculation, a narrow band of cells forms below the infection site, and extends along the axis of the root at a ratio of approximately 4:1 axis:circumference (Fig. 6.6). Later observations (72 hrs - Figs. 6.7 - 6.10; 96 hrs - Fig. 6.13) show development of two regions of dividing tissue. One is of cytoplasmic-rich cells directly below the infection site and, following Newcomb (1981), becomes the meristem that forms the infected nodule tissue (Figs. 6.7, 6.9, 6.10). The other region of cells connects the nodule meristem to the vascular tissue of the root (Figs. 6.7 and 6.9). These cells possess large vacuoles, and only small amounts of cytoplasm. They divide predominantly anticlinally, at least in the early stage. Fresh tissue hand sections of 96 hr material observed under FM showed that the vascular connections between the the developing nodule and the root vascular bundle develop in this second focus (data not shown).

As the nodule forms, the epidermal tissue cracks and tears (Fig. 6.13) and a brown stain appears at this point on the root surface. This is quite distinct from the browning associated with infected hairs, and probably represents the oxidised contents of broken epidermal tissue. From observations of fresh hand-cut tissue, the outer surface of the nodule was seen to be connected to the endodermis (data not shown). This phenomenon was outside the planned scope of studies and was not pursued. However, it could be important to trace the development of the outer nodule and show its derivation from endodermis of pericycle cells. This may at least partially explain the readiness for
nodulation of the zone between RT and SERH, where pericycle cells, already in division for formation of endodermal tissue, are 're-routed' to nodule epidermis. In clover, infection in mature parts of the root is slower than in the transient zone, and perhaps in these parts the pericycle tissue has to be first stimulated by the infection process, making the overall process of nodule synthesis longer. In siratro, stimulation of mature endodermal tissue to divide does not usually occur, and nodules do not form from mature regions of the root.
CHAPTER 7

MINUS-RHIZOBIM CONTROL AND ROOT HAIR DEVELOPMENT
7.1 INTRODUCTION

In all experiments, a large number of positive and negative controls were used to closely monitor the two experimental systems. In the pouch system, minus-Rhizobium (-R) controls were never infected, despite their open tops, and close inspection of the root surface at LM and often SEM and TEM for each experiment never revealed contamination. For the plate method, fungal contamination was an occasional problem, but -R plates were always found to be free of Rhizobium. Of course, -R plants were also examined for the purpose of comparison.

7.2 MATERIALS AND METHODS

7.2.1 PLANT ASSAY

Only siratro was studied in pouch and plate systems. For any given experiment, -R controls were treated exactly as experimental plants. 50% of -R controls were inoculated with sterile water, the rest uninoculated. For micro-inoculation procedures, some -R controls had beads attached, some beads + sterile water and the remainder were left to grow in the pouch untouched. Pouches for -R controls were marked at RT in a similar manner to experimental pouches, and tissue in this area was examined thoroughly at a later stage. However, to be sure of contamination-free plants, -R controls were routinely examined along the whole length of the root by LM, and random samples were taken for SEM and TEM.
7.2.2 MICROSCOPY

LM, SEM and TEM (including freeze-substituted material) was used according to details outlined in Chapter 2. For root hair emergence studies (using Nomarski optics), thin longitudinal sections of fresh root material between RT and emerged root hairs were cut using a double-edged razor blade. The resulting 3-4 cell thick slices greatly enhanced viewing of the emerging hair.

7.3 RESULTS AND DISCUSSION

7.3.1 ROOT HAIR EMERGENCE

Any continual observations of a particular hair over a long period were impossible due to the slicing technique. To be certain of the sequence of events in this case, a large number of specimens were examined (>50 plants) and representative micrographs of the stages in root hair development are presented in Fig. 7.1. Root hairs are known to commence development just before the trichoblast finishes elongation (see Bauer 1981). At this stage in siratro, the nucleus of the cell is more-or-less centrally located below the upper surface of the trichoblast (Fig. 7.1A). The initial protrusion of the root hair develops directly over the nucleus (Fig. 7.1B). It would seem, therefore, that there is nuclear involvement in the commencement of root hair growth. It is known that various enzymes are active in the trichoblast prior to root hair outgrowth (Dosier and Riopel 1977). It is interesting that Dosier and Riopel demonstrated that the activity of only one enzyme, phosphorylase, increased just prior to and during root hair outgrowth. They suggested that root hair development begins
at a point marked by elevated phosphorylase activity. As phosphorylase is known to degrade starch into sugars (usually glucose units) it would be interesting to know if a similar enzyme is active in siratro, producing the galactose units that were detected by RCA I lectin on siratro root hair tips (Chapter 8).

Within 1.5 hrs of root hair initiation, the nucleus moves to the lower part of the trichoblast, leaving a thin layer of cytoplasm below the emerging tip (Fig. 7.1C). Cytoplasmic threads are maintained between nucleus and tip. These initial sequences are relatively slow compared to growth of an emerged and growing hair. Accurate measurements of the timing of these events were not made. However, the series in Figs. 7.1A-7.1C represent approximately 1.5 hrs. By approximately 2 hrs after root hair initiation (Fig. 7.1D), the nucleus commences to re-migrate to the tip, maintaining a small distance behind it (Figs. 7.1E and 7.2F). Hairs always grow out perpendicularly from the root surface (Fig. 7.2) unless disturbed mechanically. If a hair touches a surface (such as a micro-inoculation bead) it will follow that surface (see Fig. 6.13).

7.3.2 TIP GROWTH

The freeze-substitution (FS) technique is excellent for the preservation of surface cells such as root hairs. Cells deeper in tissue tend to suffer from ice damage, and because of this FS is limited in its use for infection studies. Observations of root hair FS material (Fig. 7.3) showed a vacuolate cell with cytoplasm and nucleus localised in the hair. The hair tip is a concentration site for vesicles that contribute to tip growth (Fig. 7.4, see Chapter 4), the vesicles meet the hair tip wall and break open, contributing wall
and membrane material. The vesicles may also contain the b-D-Galactose that was detected at the root hair tip surface by the lectin RCA I (Chapter 8). The hair tip dome (Fig. 7.4) is quite hemispherical in shape. Grazing sections of root hair tips of other examples (Fig. 7.5) show that microtubules are involved in the growth processes. The many vesicles that contribute to tip growth appear to arise from dictyosomes, which are prominent near the tip region (Fig. 7.5). Transverse sections of hairs away from the tip region in a vacuolated area (Fig. 7.6) reveal a cytoplasm rich in RER, mitochondria and amyloplasts, as well as dictyosomes.

Preservation of material by conventional fixation was not as good as by FS. In addition, plasmolysis tended to occur in varying amounts. High magnification of hair walls (Fig. 7.7) showed that blister-like formations can occur containing plasmalemmasome-like material, which are finger-like projections of the plasma membrane. These phenomena also occur in infected tissue (see Chapter's 4-6, and 11) and cannot, therefore, be attributed to infection. However, the large interfacial zones attributed to infection were not found in uninfected controls.

7.3.3 THE EPIDERMAL SURFACE

When viewed by SEM, uninfected tissue appears smooth. There are no erosional areas or holes as occurs in infected tissue (Fig. 7.8 and 7.9; compare to Chapter 4). Remnants of root cap slime between epidermal cells cannot be detected by SEM, but are easily visible using CON A or RCA I lectin (see Fig. 8.2)(see also Hinch & Clarke 1980). However, occasional remnants of root cap slime are left on root hair surfaces or on the epidermis, and care was taken not to confuse such remnants with inoculum material.
LECTIN STUDIES OF PLANT AND BACTERIAL SURFACES
Research in the last 10 years has disclosed the wide-spread biological importance of cell-surface carbohydrates in the many processes involving specific cellular recognition (see eg Sharon & Lis 1981; Turner 1980). These findings and the realization that relatively small changes in the structure of complex surface carbohydrates may lead to large effects on recognition specificity (eg Stanley & Sudo 1981) and that more structural variation can be contained in complex carbohydrates, on a weight basis, than in either proteins or nucleic acids (Sharon & Lis 1981) have stimulated considerable interest in cell surface carbohydrates. Since plant-parasite and Rhizobium-legume interactions represent well-defined examples of specific recognition, it has been suspected that surface carbohydrates on the cells of these organisms may be functionally involved.

Based on these considerations and a small amount of direct evidence, several authors have proposed elicitor-receptor models to explain the recognition of incompatible but not compatible pathogen races or strains by plant cells (see eg Albersheim & Anderson-Prouty 1975; Keen 1982a). The models generally propose that incompatible pathogen races or strains possess surface molecules, probably complex carbohydrates, which interact with surface receptors on plant cells and thereby lead to stimulation of active plant defence mechanisms. Compatible pathogen races or strains are postulated to have structurally altered surface molecules that are not efficiently recognized by the plant receptors. Some experimental evidence supports this model with fungus-plant systems (Anderson 1980; Keen 1982b; Wade & Albersheim 1979) but it is, as yet, inconclusive. The model may also have
applicability to plant recognition of incompatible bacteria, and tests of its occurrence in bacterial systems would be desirable.

8.1.1 RHIZOBIUM-LEGUME INTERACTIONS

In their landmark paper, Bohlool & Schmidt (1974) reported a strong correlation between the in vitro binding of soybean seed lectin to *Rhizobium japonicum* cells and the ability of such strains to infect soybean roots. These observations were independently confirmed by Bhuvaneswari et al. (1977) who also demonstrated that the soybean lectin haptens, N-acetyl-D-galactosamine and D-galactose, reversed lectin binding to *R. japonicum* cells. It was later demonstrated by Stacey et al. (1980) that the soybean lectin haptens specifically inhibited attachment of *R. japonicum* cells to soybean roots. In early studies, a few infective strains of *R. japonicum* were found that did not bind soybean lectin (Bohlool & Schmidt, 1974; Bhuvaneswari et al. 1977) but later work by Bhuvaneswari & Bauer (1978) demonstrated that the few non-lectin-binding *R. japonicum* strains in fact possessed lectin binding properties when cultured in a soybean root exudate medium.

Lectin-mediated attachment has also been implicated in clover-*R. trifolii* interactions and perfect correlations between in vitro lectin binding and strain infectivity were described (Dazzo & Hubbell, 1975). The hapten 2-deoxyglucose, was shown to specifically prevent lectin binding to bacterial cells and also prevented the attachment of *R. trifolii* cells to clover root surfaces. Significantly, the clover seed lectin originally used in binding studies has been shown to be present on the root hair surfaces where *Rhizobium* attachment occurs (Dazzo et al. 1978). Similarly soybean seed lectin has recently been
localized to the site of Rhizobium attachment to roots (Stacey et al. 1980; Stacey & Brill 1982).

In spite of the mounting evidence indicating lectin involvement in specific attachment, some evidence argues against this mechanism for conferring host range specificity (see Bauer 1981; Stacey & Brill 1982). Recent models de-emphasize the lectin hypothesis and instead propose that some plant receptor, which may or may not be lectin, recognizes a specific component on the bacterial surface, possibly resulting in receptor-induced responses in one or both members of the symbiotic pair. This concept, in several forms, is discussed in a recent review by Bauer (1981). Stacey & Brill (1982) present the additional possibility that lectins may function to increase the affinity of a host recognition process that does not itself involve lectin.

Whether involved in lectin binding or not, Rhizobium cell surface carbohydrates are being scrutinized closely to determine whether they are specifically recognized by legume cell-surface receptors (see Bauer 1981). The structure and biological activity of Rhizobium exopolysaccharides, capsular polysaccharides, lipopolysaccharides, and unique bacterial glucans are being examined (B.G.Rolfe pers. comm.).

The biological importance of any particular carbohydrate component remains a subject of controversy, but current indications suggest a role for EPS in the soybean-R. japonicum interaction (Bauer 1981). The study of Rhizobium carbohydrates has increased our knowledge of bacterial surface architecture and will continue to provide important insight for the question of how bacteria are recognized by host plants. Recent reports that the host range of Rhizobium spp. may be determined by plasmid-borne factors (Beynon et al. 1980; Brewin et al. 1982).

8.1.2 LECTINS AS PROBES

Apart from their hypothetical involvement in recognition and specificity, lectins are also useful tools in biology because of their highly specific recognition of sugar molecules. Using lectins with differing affinities, a surface carbohydrate profile of any cell can be obtained. Until recently, this kind of work was tedious and expensive. However, a 'kit' of lectins recently available from the U.S.A. (see below) has made this kind of work quick, easy and relatively cheap, and is excellent for separating organisms that may differ by only a single surface sugar. In this Chapter, experiments are presented in which a number of *Rhizobium* mutants and the *Siratro* root surface were probed with lectins.

8.2 MATERIALS AND METHODS

8.2.1 LECTINS

All lectins were purchased from E-Y Laboratories, Inc., U.S.A. Lectins were supplied labelled with FITC as 1mg lectin in 1ml of buffer, all lectins were diluted to 200ug.ml⁻¹, divided into 0.5ml aliquots, and maintained at -20°C.
<table>
<thead>
<tr>
<th>LECTIN</th>
<th>SOURCE</th>
<th>HAPITEN</th>
<th>BUFFER</th>
</tr>
</thead>
<tbody>
<tr>
<td>PNA</td>
<td><em>Arachis hypogaea</em></td>
<td>D-Gal-b(1-3)GalNAc*</td>
<td>PBS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Use Galactose or GalNAc)</td>
<td></td>
</tr>
<tr>
<td>WGA</td>
<td><em>Triticum vulgaris</em></td>
<td>Sialic acid</td>
<td>PBS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Use chitobiose or GalNAc</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>or Di-Acetyl-D-Glucosamine)</td>
<td></td>
</tr>
<tr>
<td>SBA</td>
<td><em>Glycine max</em></td>
<td>GalNAc or D-Galactose</td>
<td>PBS</td>
</tr>
<tr>
<td>UEA-I</td>
<td><em>Ulex europaeus</em></td>
<td>a-L-Fucose</td>
<td>PBS</td>
</tr>
<tr>
<td>MPA</td>
<td><em>Maclura pomifera</em></td>
<td>Galactose</td>
<td>PBS</td>
</tr>
<tr>
<td>DBA</td>
<td><em>Dolichos biflorus</em></td>
<td>GalNAc</td>
<td>PBS</td>
</tr>
<tr>
<td>GSA-I</td>
<td><em>Griffonia simplicifolia</em></td>
<td>a-D-Galactose</td>
<td>PBS</td>
</tr>
<tr>
<td>GSA-II</td>
<td><em>Griffonia simplicifolia</em></td>
<td>GlcNAc**</td>
<td>PBS</td>
</tr>
<tr>
<td>RCA-I</td>
<td><em>Ricinus communis</em></td>
<td>b-D-Galactose</td>
<td>PBS</td>
</tr>
<tr>
<td>CON A</td>
<td><em>Canavalia ensiformis</em></td>
<td>a-L-Mannose or a-L-Glucose</td>
<td>TRIS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Use a-Methyl-Mannoside)</td>
<td></td>
</tr>
</tbody>
</table>
PBS: Phosphate buffered saline

\[ \begin{align*}
\text{NaH}_2\text{PO}_4 & \quad 0.43 \text{ g} \\
\text{Na}_2\text{HPO}_4 & \quad 1.48 \text{ g} \\
\text{NaCl} & \quad 7.20 \text{ g}
\end{align*} \]

to make a 1 litre solution

TRIS: TRIS

\[ \begin{align*}
\text{TRIS} & \quad 0.61 \text{ g} \\
\text{CaCl}_2 \cdot 2\text{H}_2\text{O} & \quad 0.15 \text{ g} \\
\text{NaCl} & \quad 0.88 \text{ g} \\
\text{MnCl}_2 \cdot 4\text{H}_2\text{O} & \quad 0.20 \text{ g} \\
\text{MgCl}_2 \cdot 6\text{H}_2\text{O} & \quad 0.20 \text{ g}
\end{align*} \]
pH 7.0

to make a 1 litre solution

Dissolve TRIS in 70% of total solution, use HCl to bring to approximate pH, dissolve Ca, Mg, Na, and Mn in rest of solution, add to buffer while stirring, correct pH with HCl, (otherwise Mn precipitates to form Mn(OH)_2).

\*GalNAc: N-Acetyl-α-D-Galactosamine

\*\*GlcNAc: N-Acetyl-α-D-Glucosamine

8.2.2 BACTERIAL STRAINS

Strain ANU240 (see Chapter 4)

Strain ANU265 (see Chapter 9)

Strain ANU1255 (see Chapter 9)

Strain ANU2861 (see Chapter 11)
Control strains: strain USDA 110 (*R. japonicum*) positive for SBA lectin (Bohlool & Schmidt 1974); *Saccharomyces cerevisae* positive for CON A (Clarke & Hoggart 1982).

8.2.3 PLANT ASSAY

Pouched plants of siratro and *Zea mays* were grown until 4-5cm of root was available. The upper part of the plant was removed, roots were used either unwashed or were washed in buffer appropriate to the lectin to be used. *Zea mays* is a positive control for UEA lectin (Hinch & Clarke 1980).

8.2.4 PROBING RHIZOBIUM

All *Rhizobium* strains were tested at various stages of growth (from early log to stationary, i.e.: 12, 19, 21, 24 and 48 hrs) using either BMM or TY media. Cells were either washed or unwashed. If washed (in appropriate buffer) pellets were resuspended in the appropriate buffer for each lectin. All lectins were used at a concentration of 200μg.ml⁻¹, as recommended by the supplier. 100μl of lectin solution was incubated in the dark for 0.5-1hr with either 200μl of living rhizobia, or heat-dried cells on a microscope slide, at room temperature. For hapten controls, lectins were first incubated in the appropriate 0.2M sugar solution for 20min before adding to the various *Rhizobium* strains. All experiments were repeated twice.

8.2.5 PROBING THE PLANT SURFACE

Roots were incubated in the dark in a 100μg.ml⁻¹ solution of lectin for 0.5-1hr at room temperature. For hapten controls, lectins were pre-incubated for 20min in the appropriate 0.2M sugar solution before applying to the root surface.
8.2.6 MICROSCOPY

Rhizobia were mounted on microscope slides directly from the incubated mixture; washing of the cells was found to be unnecessary. Plant material was either un-rinsed or rinsed three times with appropriate buffer, and mounted in buffer on a microscope slide. Material was observed using FM at either U420 or B515 settings (see Chapter 2) which are appropriate for the proper excitation of FITC.

8.2.7 INHIBITION EXPERIMENT

The micro-inoculation procedure was used (Chapter 2). Plants were micro-inoculated (marked by an ion exchange resin bead) with either 50, 100 or 200 μg.ml⁻¹ RCA I lectin using a large amount (ca. 100 nl). This was incubated for one hour, and then a small inoculum (ca. 20 nl, containing approximately 500-1,000 cells) of strain ANU240 was micro-inoculated at the same position. Flood and micro-inoculated strain ANU240 and uninoculated plant controls were used.

8.3 RESULTS

8.3.1 RHIZOBIUM STRAINS

No autofluorescence was found with living rhizobia, but a slight blue autofluorescence occurred with heat-dried cells, this was too weak to photograph.

In all trials, the control SBA on USDA110 bound to >90% of cells, in a manner similar to that described by Bohlool and Schmidt (1974) who first described binding for soybean. The hapten-SBA incubated control completely eliminated binding to the cells. For the control CON A on S. cerevisiae, binding occurred to >60% of the yeast cells, the hapten
eliminating this binding. These results gave assurance that the methodology was working.

In the first of the three trials, the lectin UEA bound strongly to joined 'twin' cells (presumably cells just after division) of the strains ANU265 and ANU1255, but this result did not occur again when the experiment was repeated twice. Otherwise, lectins did not bind to any strains under any test conditions. CON A bound to residues in the medium of unwashed cells, due to the presence of mannitol in the medium.

8.3.2 PLANT SURFACES

Both plants had a faint blue autofluorescence, making the bright green FITC fluorescence easy to distinguish. The autofluorescence of siratro is so faint that the Nikon photographic equipment was unable to detect any light emitting from its surface. Long exposures resulted in blank film. Thus, Figures of controls are not included.

The control UEA on Zea mays (Hinch and Clarke 1980) showed slight binding to root cap slime and slime between epidermal cells. Hapten controls eliminated this binding.

The results of lectin probing on siratro by the various lectins were more interesting than on the Rhizobium strains, and are summarized as follows: (binding scaled subjectively 1-5 from weak to strong, depending on level of FITC fluorescence)

WGA very good slime binding (=3) at root cap and slight binding (=1) between epidermal cells along elongation zone and first part of SERH.

PNA similar to WGA.
CON A  similar to WGA but binding =4 at root cap, and =3 along elongation zone.

RCA I  strong slime binding (=5) at root cap (Fig. 8.1) and along the elongation zone into the first part of the SERH (Fig. 8.2) and also at the root hair tips (Figs. 8.3-8.7).

MPA, DBA, GSA I, GSA II, UEA, and SBA all showed no binding to the surface of siratro.

All hapten controls worked very well for all positive results, eliminating binding completely. CON A and RCA I still showed good fluorescence at 50ug.ml\(^{-1}\) (Fig. 8.4) but lower fluorescence levels at 20ug.ml\(^{-1}\).

RCA I bound to root hair tips so well that it was possible to detect emerging root hairs by RCA I-binding before they could be detected by either bright field or Nomarski optics (Figs. 8.5-8.7). There was an even binding distribution of RCA I to emerged root hair tips compared to emerging hairs when lectin levels were applied at 50ug.ml\(^{-1}\) (compare Figs. 8.4 and 8.7 to Fig. 8.3). High levels of lectin resulted in large 'clumps' of fluorescing material at the root hair tips. RCA I did not aggregate uniformly over emerging root hair tips (Figs. 8.5-8.7). Generally, RCA I bound to emerging and developing root hairs better than fully grown hairs in the MRHZ.

8.3.3 INHIBITION EXPERIMENT

Because of the implications of RCA I specific sugars being involved in recognition and/or attachment of Rhizobium to the root hair tip, and in light of the results of Hinch and Clarke (1980), where UEA lectin
prevented attachment of Phytophthora sp. zoospores to Zea mays root in the elongation zone, an inhibition experiment was conducted to establish if RCA I-binding to root hair tips prevents infection by Rhizobium by saturating such hypothesized recognition or attachment sites. The results are presented as follows:

PLANTS NODULATED (numbers in brackets represent a second trial)

<table>
<thead>
<tr>
<th>LECTIN ADDED</th>
<th>RCA 100</th>
<th>RCA 200</th>
<th>240 micro</th>
<th>240 flood</th>
<th>uninoc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>plants with nodule equal to wild type</td>
<td>3 (2)</td>
<td>3 (3)</td>
<td>29 (26)</td>
<td>30 (29)</td>
<td>- (-)</td>
</tr>
<tr>
<td>plant deaths</td>
<td>- (1)</td>
<td>2 (1)</td>
<td>- (2)</td>
<td>- (1)</td>
<td>- (2)</td>
</tr>
<tr>
<td>plants that didn't nodulate</td>
<td>27 (27)</td>
<td>25 (26)</td>
<td>1 (2)</td>
<td>- (-)</td>
<td>15 (13)</td>
</tr>
</tbody>
</table>

TOTAL 30 (30) 30 (30) 30 (30) 30 (30) 15 (15)

These results show that pre-incubation of the root with RCA I lectin at either 100 or 200ug.ml\(^{-1}\) prevented infection by the wild type strain ANU240. The low number of plants nodulated can be considered 'noise' in the system. Note that micro-inoculation and flood
inoculation of siratro by strain ANU240 produced almost 100% nodulation of plants.

LM observations showed no attachment of rhizobia at the lectin inoculated sites in a way normal to strain ANU240, though they were present on the root surface. The lectin did not prevent root hair growth (Fig. 8.8) and the tissue did not brown at the inoculation point. There was no root hair curling observed. RCA I did not appear to affect living strain ANU240 cells, even when incubated overnight in a concentration of 100ug.ml⁻¹ RCA I, the culture still appeared actively mobile.

8.4 DISCUSSION

The results presented above demonstrate that different sugars may be present on the siratro root surface. These sugars are:

sialic acid (WGA)
D-Gal-b(1-3)GalNAc (PNA)
a-L-Mannose or a-L-Glucose (CON A)
b-D-Galactose (RCA I)

In addition, the lectin RCA I binds to the root hair tips, implying that the sugar b-D-Galactose is present. This binding to the root hair tips prevents normal infection by strain ANU240 without apparent damage to either plant or rhizobia. The change in binding characteristics of RCA I from emerging to more mature root hair tips may reflect a transition in intensity of the b-D-Galactose at the hair tip. Why there is uneven distribution of the sugar at the emerging
hair tips is unknown, though it could occur through a pulsing production as the mechanisms for the sugars' production commences. In any case, there is no doubt that the presence of β-D-Galactose at the site of emerging hairs could act as a target for rhizobia, enabling them to establish on the hair-forming site even before the hair has emerged.

It is unfortunate that no lectin bound reliably to any of the bacterial strains tested (though controls worked well), other lectin sources are now becoming available for further testing. It is possible that no binding occurred because strain ANU240 has recently been found to have no capsule (John Redmond pers. comm.). Follow-up experiments should include pre-incubation of strains on root surfaces prior to testing with lectin probes, as well as incubation of strains with a variety of sugars to search for sugar-mediated responses such as agglutination of cells that would imply recognition.

It is critical that more be known as to the nature, distribution, and sugar binding properties of lectins in the roots, and of the relationships between root and seed lectins. Even with a highly specific recognition system functional at the nodulation site, other events are required to communicate the success of the recognition so as to signal the next step toward entry into the root tissue. At a more preliminary but equally critical stage it must be determined if the plant does indeed excrete signal compounds or special substrates that selectively favour appropriate rhizobia, and somehow accomplish this in the competitive, microbe-rich environment of the rhizosphere.
From a genetic point of view, an immediate approach is the use of transposon insertion mutations (see Chapter 1). It would seem feasible to screen for transposon mutations of bacterial genes conferring specific incompatible responses in host plants. Because they carry drug resistance markers, transposon insertions are easy to detect and the mutants could be screened for altered host reactions. The decisive final steps would involve comparison of the surface carbohydrate structure of the wild-type compatible organism with its incompatible mutant derivative, and assay of their glycosyl transferase enzymes. It is possible that a host specificity gene, for example, would code for a glycosyl transferase enzyme, lacking in the wild-type, and would produce a unique surface carbohydrate structure.
CHAPTER 9

MUTANT STUDIES 1: STRAINS ANU265 AND ANU1255

9.2 MATERIALS AND METHODS

9.2.1 BACTERIAL STRAINING

Strain ANU265 - the plant disease derivative of strain ANU129 (Morrison et al., 1981).

Strain ANU125 - strain ANU265 carrying a chromosome in the nucleotide region of the gyrA plasmid (Morrison 1964).

Control: strain ANU265 attenuated.

9.2.2 PLANT ASSAY

Strains were studied by the four-week bean plant assay and wet-dry incubation (see Chapter 8).

9.2.3 MICROSCOPY

Ten plants from ten pots per treatment were sprayed with water and prepared or processed for TEM by the schedule and not in contrast. Treatments were assigned for TEM. Timing of observation and conclusion was every 3 hrs for the first 12 hrs after incubation. At the 12th hour, the plants were collected and then daily or twice daily after incubation.
9.1 INTRODUCTION

Two Hac^- Nod^- mutants were studied by light and electron microscopy to discover any root surface activities. Even though these strains are apparently unable to infect siratro, it was thought possible that certain activities, such as the random enzymic attack that is associated with parent strain ANU240 infection of siratro, may be present.

9.2 MATERIALS AND METHODS

9.2.1 BACTERIAL STRAINS

Strain ANU265 - Sym plasmid-cured derivative of strain ANU240 (Morrison et al. 1983).

Strain ANU1255 - strain ANU240 carrying a single Tn5 in the nodulation region of the Sym plasmid (Morrison 1984).

Controls: strain ANU240, uninoculated.

9.2.2 PLANT ASSAY

Strains were studied by the pouch method, both flood and micro-inoculated (see Chapter 2).

9.2.3 MICROSCOPY

Ten plants from ten pouches per treatment per strain were fixed and embedded or prepared for SEM by the schedules set out in Chapter 2. Fresh material was observed for LM. Timing of observation and fixation was every 2 hrs for the first 12 hrs after inoculation, at 24 hrs and then daily up to seven days after inoculation.
9.3 RESULTS

In all respects, the two mutants shared the same characteristics and will be described together. Comparison was made with uninoculated material (Chapter 7) and parent strain ANU240 (Chapters 4-6). When examined under LM, attachment of the mutants to root surfaces appeared similar to strain ANU240, including polar attachment (data not shown). However, the mutant rhizobia did not move away from the micro-inoculated areas, even after seven days. In marked contrast, strain ANU240 bacteria tended to move away from the micro-inoculated area when micro-inoculated under exactly the same conditions (growth phase, cell density, plant age, time of day) (Fig 9.1). Thus, a distinct colony formed at the micro-inoculation area for both mutants. Under SEM, these colonies were seen as clumps or aggregates of bacteria (Fig. 9.2). SEM of strain ANU240-inoculated material showed little or no aggregation (Fig. 9.3). Twenty samples each of fresh material and material prepared for SEM were studied to ensure that these phenomena were consistent.

FM studies with acridine orange staining showed no stimulation of cell division below the inoculated sites.

TEM and SEM studies showed no 'enzymic' erosion of the epidermal or hair walls (Figs. 9.2, 9.4-9.6; compare to Figs. 4.12-4.16). Mutant cells contained large amounts of PHB compared to the strain ANU240 control of comparable age. The mutant bacteria also tended to take on exaggerated elongated shapes (Fig. 9.4) compared to the more 'rounded' form of strain ANU240.

A summary of the main features of these strains compared to the wild type strain (ANU240) and mutant strain ANU2861 (Chapter 11) is presented in Table 9.1.
9.4 DISCUSSION

Loss of the Sym plasmid (strain ANU265) results in complete loss of symbiotic capacity and certain root surface activities. However, such information is really only of general use, because of the large number of genes carried on the plasmid. Strain ANU1255, on the other hand, has a Tn5 located in a particular gene in the Sym plasmid (in the Nod region) and therefore the phenotype of the strain is defined by the loss of that gene. In fact, loss of this particular gene confers a similar phenotype to that associated with complete loss of the Sym plasmid, and demonstrates the possible importance of sequential expression of genes in the Sym plasmid, where non-expression of one gene will prevent expression of 'downstream' genes. It is not known if interruption of a downstream gene will also prevent colonization, random epidermal erosion and infection, because it is not known if such genes need to act in concert. Thus, interruption of a gene further along the plasmid from the strain ANU1255 gene may still have the same phenotype as strain ANU265, and it may be impossible to isolate mutants that are wild-type in respect to colonization and erosion, but are Hac-. Although entirely hypothetical, such probabilities should be considered.

It is interesting that these two mutants are characteristic of the avirulent bacteria described by Huang et al. (1974) which did not move more than a few mm from the point of inoculation. In the case of these mutants derived from strain ANU240, the extent of movement is much less than that of strain ANU240, most likely only a few hundred microns. The work of Huang et al. (1974) also showed that their avirulent bacteria became pleiomorphic and formed bacterial clumps,
appearing as though an agglutination-like reaction had occurred in vivo. The mutant strains ANU265 and ANU1255 show similar changes in shape and a tendency to clump and remain at the original inoculation area. It is known that glucosides, which are not toxic themselves, break down to release highly toxic 'aglycones' (Anderson 1982). Any bacteria producing the necessary glucosidase enzyme may therefore contribute to its own downfall. Thus, one of the many possible explanations for the phenotypes of strains ANU265 and ANU1255 is that they are derepressed for glucosidase production.

The results suggest that the two mutants lack certain enzyme capabilities and are unable to move through the mucigel that exists at root surfaces (Adrienne Clarke, pers. comm.). Their inability to erode the root surface walls and hairs also suggests that enzyme-encoding gene(s) are not expressed. Thus, loss of a single gene within the Sym plasmid can completely preclude symbiotic activity, and emphasizes the highly sensitive nature of the symbiotic sequence. These results also suggest that host specificity can be explained by single gene differences, as occurs between A and O blood groups (Furth 1980). It is suggested that the gene expression lost in the mutant strain ANU1255 may be responsible for the initial perturbation of root hair tip growth, and that such genes are also responsible for movement through root surface mucigel and the occurrence of random erosions in the root surface.
<table>
<thead>
<tr>
<th>STRAIN</th>
<th>ABILITY TO SWIM IN MEDIA</th>
<th>COLONIZATION OF ROOT SURFACE</th>
<th>EROSIONS &amp; HOLES</th>
<th>CURLING (Hac)</th>
<th>NODULATION (Nod)</th>
<th>STIMULATION OF CORTICAL CELL DIVISION</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANU240</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ANU265</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ANU1255</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ANU2861</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

TABLE 9.1
CHAPTER 10

MUTANT STUDIES 2: STRAINS ANU265(H1) AND ANU265(XA1)

DNA fragments in the Tn5 transposon insertion in the sym plasmid of strain ANU265 were chosen and used to replace the wild-type equivalent fragments. These were transformed into the sym plasmid-free strain ANU265 by Myc, Bacto Bacti (Amersham Buchler, 0.5-0.8), on the vector plasmid pAM, and the resulting transconjugants were inoculated onto strata.

The aims of the experiments were:
1) to determine a minimal region of the sym plasmid required to initiate infection, and
2) to further characterize the phenotype of the gene disrupted by the Tn5 insertion in strain ANU265.

10.2 MATERIALS AND METHODS

10.2.1 BACTERIAL STRAINS

ANU265(XA1) - carrying the 5.4kb, flanking fragment on the pAM vector in low copy number (Fig. 10.1).

ANU265(H1) - carrying the 1.8kb, minimal fragment on the pAM vector in low copy number (Fig. 10.1).

Control strains ANU240, ANU261, ANU250-1 and untransformed plants.

10.2.2 PLANT HOSTS

Strata was micro- and flood-inoculated using host plants from two 'pouches per treatment' (half the number for controls which were flood inoculated only). The experiment was repeated three times; the last two repeats using strata re-sealed by Mr. Bax.
10.1 INTRODUCTION

DNA fragments containing the gene(s) inactivated by the Tn5 insertion in the Sym plasmid in strain ANU1255 were cloned and used to isolate the wild-type equivalent fragments. These were transferred to the Sym plasmid-cured strain ANU1255 by Mr. Brant Bassam (Genetics Department, R.S.B.S.), on the vector plasmid RP4, and the resulting transconjugants were inoculated onto siratro.

The aims of the experiments were:

i) to determine a minimal region of the Sym plasmid required to initiate infection, and

ii) to further characterise the phenotype of the gene disrupted by the Tn5 insertion in strain ANU1255.

10.2 MATERIALS AND METHODS

10.2.1 BACTERIAL STRAINS

ANU265(XA1) - carrying the 16kb XhoI fragment on the RP4 vector in low copy number (Fig. 10.1)

ANU265(H1) - carrying the 6.7kb HindIII fragment on the RP4 vector in low copy number (Fig. 10.1)

Controls: strains ANU240, ANU265, ANU1255 and uninoculated plants.

10.2.2 PLANT ASSAY

Siratro was micro- and flood-inoculated using ten plants from ten pouches per treatment (half the number for controls which were flood inoculated only). The experiment was repeated three times, the last two repeats using strains re-cloned by Mr. Bassam.
10.2.3 MICROSCOPY

LM and FM only were used. Due to cloning problems, in which insufficient numbers of transconjugant clones were grown, SEM and TEM work was not done. Studies are in progress by Mr. Bassam to improve copy number, transfer and cloning procedures. Plants were examined every 2 hrs after inoculation for the first 12 hrs, at 24 hrs, and then daily for 1 week.

10.3 RESULTS

Both strains affected the area around the first emerging laterals below the RT mark by a browning of the tissue around the emergence point of these laterals (Fig. 10.2). This response occurred within 24 hrs in over 90% of plants examined (30 plants in 10 pouches per strain).

The transconjugant strain ANU265(XA1) showed a small number of curled root hairs (Fig. 10.3) after 3 days, but no attempt was made to objectively assess this curling because of the low number of transconjugants in the inoculum. These curls occurred in the browned region only, and mostly on the emerged lateral rather than on the tap root. The curled hairs did not brown at their surface (see strain ANU240 response, Chapter 6) and no cell division was detected below the curling sites when tissue was examined under Nomarski optics or stained with acridine orange and viewed under FM. Nuclei and nucleoli appeared to be at resting stage. The transconjugant strain ANU265(H1) showed no sign of curling or cell division.
10.4 DISCUSSION

From this preliminary work some indications for future work and results have been observed. Both the transferred fragments are able to stimulate a general 'browning' response from the plant. This response is not seen in any of the controls, including strain ANU240. The browning effects seen by inoculation of strain ANU240 are completely different from this result. It is possible that this particular browning response is the result of each strain causing a sensitive response by the plant at an area of surface damage (where laterals have torn the epidermal tissue). The HindIII fragment is unable to elicit further responses. However, the Xhol fragment is able to induce a low frequency of hair curling. The curled hairs do not have the distinct browning that is thought to be caused by the thickening of the outer wall of the hair, and may thus represent a stage between Hac\textsuperscript{−} and the phenotype of strain ANU2861 (Chapter 11) which curls, causes thickening, and partially penetrates hairs at the infectible zone. The position and timing of the curling caused by the Xhol fragment (on the lateral root) indicates a slow response by the mutant, which may again reflect the low incidence of the clone.

This experiment has given good indication that cloning of small regions of the Sym plasmid will give some answers to their various encoded functions. It is clear that the 6.7 kb HindIII fragment has insufficient information to initiate the symbiosis; although it is uncertain how many genes are on this fragment. The Xhol fragment, however, clearly has enough information to curl some root hairs, though it is not a generalised curling phenomenon. It is known that this 16kb fragment contains the genes \textit{nodD} and a host specificity
gene, and possibly parts of the nodABC gene group (B.G. Rolfe, pers. comm.). More intensive studies are needed to answer detailed questions such as the occurrence of penetration and plant responses.
With the exception of adenine, the other studies of auxotrophic mutants of Rhizobium japonicum studied by Hale (1979) were consistently symbiotically defective. Another auxotrophic wild-type mutant, determined by Schwinghammer (1980) and Adelsbach & Schwinghammer (1979), however, a large proportion of the other studies of auxotrophs, such as methionine, tryptophan and folate auxotrophs, were described by Hale as giving rise to effective nodules containing protoplasts. These mutants were clearly formed by infections. Other auxotrophic acts requiring mutants may be symbiotically defective as a secondary consequence of the auxotrophic marker, and protoplast-to-
revertants have restored symbiotic competence. It is known from the studies by Schwinghammer (1978) and Humbert and Schwinghammer (1974) that it is possible that adenine is required for the establishment of nitrogen-fixing symbiosis but, unlike histidine, exogenously supplied adenine probably does not enter the plant tissue in concentrations sufficient to restore the symbiotic fitness of adenine auxotrophs, or may enter it in an unusable form. Adenine auxotrophs of R. meliloti are able to form nodules, but they are ineffective (Schenorr and Denaro 1972).

Despite these studies, very little has been done microscopically on nodules of the mutants. This chapter describes the phenotype of an adenine-deficient mutant.
11.1 INTRODUCTION

With the exception of adenine auxotrophs, none of the other classes of auxotrophic mutants of *Rhizobium leguminosarum* studied by Pain (1979) were consistently symbiotically defective. Adenine auxotrophs were noninfective, as determined by Schwinghamer (1969) and Pankhurst & Schwinghamer (1974). However, a large proportion of the other classes of auxotrophs, such as methionine, tryptophan and leucine auxotrophs, were described by Pain as giving rise to effective nodules containing prototrophs. These nodules were clearly formed by revertants. Thus, many amino acid-requiring mutants may be symbiotically defective as a secondary consequence of the auxotrophic marker, and prototrophic revertants have restored symbiotic competence.

From the studies by Schwinghamer (1970) and Pankhurst and Schwinghamer (1974) it is now clear that adenine is required for the establishment of nitrogen-fixing symbiosis but, unlike riboflavin, exogenously supplied adenine probably does not enter the plant tissue in concentrations sufficient to restore the symbiotic fitness of adenine auxotrophs, or may enter it in an unusable form. Adenine auxotrophs of *R. meliloti* are able to form nodules, but they are ineffective (Scherrer and Denarie 1971).

Despite these studies, very little has been done microscopically on Nod$^-$ auxotrophs. This Chapter describes the phenotype of an adenine deficient mutant.
11.2 MATERIALS AND METHODS

11.2.1 BACTERIAL STRAINS

Strain ANU240 - see Chapter 4.

Strain ANU2861 - an Ade\(^{-}\), Nod\(^{-}\), Hac\(^{+}\) derivative of strain ANU240 isolated by Mr. Han Cai Chen (Genetics Department, R.S.B.S.), who supplied the following details:

Strain ANU2861 is a Tn5-induced, mucoid mutant of strain ANU280 (a rifampicin-resistant derivative of strain ANU240). It is an overproducer of extracellular polysaccharide (EPS) and forms very slimy, translucent colonies on a variety of carbon sources. It is also an adenine-deficient mutant. From hybridization analysis, the mutant contains a single copy of Tn5 inserted into either a 15kb EcoRI or 7.5kb ClaI fragment of chromosomal DNA. It is Hac\(^{+}\) Nod\(^{-}\) on siratro, Desmodium intortum and D. uncinatum; Nod\(^{+}\) on Lablab purpurea and Leucaena sp., but with slow nodule initiation and poor nodule development giving rise to a Nod\(^{+}\) Fix\(^{-}\) phenotype. The mutant will grow on minimal media with supplemented adenine but at a reduced growth rate and final yield compared to the parent strain ANU240. When various concentrations of adenine (5mg to 20mg per litre, filter sterilized) were added to the plant growth medium, the mutant still did not nodulate siratro, D. intortum or D. uncinatum. These levels of adenine did not affect normal plant growth. Similarly, the overproduction of EPS and translucency could not be corrected by the addition of adenine. Revertants of the mutant became Km\(^{5}\) Ade\(^{+}\) (having lost the Tn5), colony morphology became similar to the wild-type strain ANU240, being normal mucoid and Nod\(^{+}\) on all test legumes. Some Ade\(^{+}\) revertants maintained Km\(^{r}\), and on hybridization analysis the Tn5
was found to be present but had translocated to a different part of
the genome. It is thought that the Tn5 insertion in strain ANU2861 is
located in an adenine biosynthesis gene exerting a pleiotropic effect
on an EPS gene.

11.2.2 PLANT ASSAY
For ultrastructural studies siratro plants were grown in pouches
(Chapter 2); ten plants per treatment were examined.
For mixed inoculum experiments, four pouches per treatment, three
plants per pouch were inoculated with 200 ul of stationary phase
culture. Fresh cultures were started ahead of schedule to coincide
with inoculation times (see Table 11.1)

11.2.3 MICROSCOPY
For ultrastructural studies, all material was fixed at 72h after
inoculation, and fixed and embedded according to the standard schedule
in Chapter 2.

11.3 RESULTS

11.3.1 INVASION OF THE INFECTIBLE ZONE: INFECTION IN HAIRS CURLED
CLOSE TO THE ROOT SURFACE
From microscopic observations, strain ANU2861 curls root hairs in a
similar manner and rate as strain ANU240 infection. The outer surface
of the curled hairs take on a brown colouration within 24 hours of
inoculation (Fig 11.1) - a characteristic of strain ANU240 infections.
A low magnification TEM of an example hair infected by strain ANU2861
(Fig. 11.2A) shows the nucleus to be in the basal part of the hair
cell, away from the infection site. In successful infections with strain ANU240, the nucleus is always very close to the penetration site. The site of penetration of strain ANU2861 in this type of infection (example enlarged in Fig. 11.2B, 11.3) is also an accumulation site for osmiophilic droplets (OPD), which can be distinguished from lipid droplets by their greater electron density. These OPD's also accumulated next to the nuclear envelope (Fig. 11.2A), though in much reduced numbers, and also occasionally around the plasmalemma of the infected cell.

An enlarged view of the infection site in this example (Fig. 11.3) reveals a complex penetration, the host walls twisted in a manner difficult to interpret. This section is considered to be at the centre point of invasion, as sections to either side of this one (Fig. 11.4) have OPD continuing all the way around the apex of penetration. Thus, the penetration area is cone-shaped, the presence of OPD truncated near the apex of the cone. This is typical of strain ANU2861 infection in the transient zone.

There are a number of variously-sized invaginations of the plasmalemma, termed 'blisters', which contain OPD and occasionally membraneous and fibrous material (Fig 11.4). These are, however, similar to blister-like invaginations in controls (see Fig. 7.7).

It is interesting that double-membrane particles, similar to those described in Chapter 5, were found to be present near the penetration site (Fig. 11.3A). An amorphous material, from which the bacteria is penetrating the cell, is completely different from the control (Chapter 5). The amorphous material has attracted electron dense particles.
The cytoplasm of the cell appears to be degenerated around the penetration site, it has none of the density and granularity found in strain ANU240 infections. There are a large number of cytoplasmic strands in the upper part of the hair cell, these have not been observed in strain ANU240 root hair invasions.

11.3.2 CELL DIVISION WITHOUT INFECTION

Although no cell division was found below the infection sites described above, areas of cell division did occur below regions without infections (macroscopic view - Figs. 11.5, 11.6). Since these studies, Calvert et al. (1984) have shown that Rhizobium japonicum is able to stimulate cell division in soybean without infection. Serial sections through one example proved there were no infection sites. Figs. 11.7-11.11 are TEM's of dividing cortical tissue, stimulated by the presence strain ANU2861. The epidermal cells shown in these Figures have become necrotic, their cytoplasm and nucleus considerably increased in electron density when compared to the dividing tissue below. However, the number of cells and the prominence of the nucleus and nucleolus in these necrotic cells indicates that they have undergone cell division prior to commencement of cell death.

Fig. 11.7B shows a band of OPD along the epidermal wall. High magnification of the epidermis (Figs. 11.8, 11.9) shows OPD concentrated in the cell walls and along the plasmalemma. A colony of strain ANU2861 above this zone of division was detected between what appears to be a dead collapsed hair cell or root cap cell and the epidermal surface (Fig. 11.10). No invasion of the tissue was detected through serial sections. However, the epidermal cell below the colony showed active signs of depositing secondary wall material.
(Figs. 11.10B and 11.11) and the colony was infiltrated with small particles, either from the host or as a result of the staining technique employed in the TEM processing. Fig. 11.11 is an enlargement of a small section of the epidermis below the colony.

11.3.3 MATURE HAIR INFECTION

An unusual feature of the mutant strain ANU2861 is its ability to infect mature (or post-emerging) hairs, an occurrence never observed with ANU240 infections. However, the frequency of such infection was not studied. Infection threads were found only in the upper part of the hair and, as the nucleus was in the basal part of the hair in this example, are presumed to have aborted prior to fixation. It is not known if this is a general occurrence, but as the mutant never nodulates, it is assumed that all infection thread invasions are eventually unsuccessful. The aborted ends of the infection threads in this example were not found. Fig. 11.12 is a low magnification TEM longitudinal section through the mature hair. The cell wall thickening of the infected cell can be compared to the uninfected cells next to it. Higher magnification (Fig. 11.13) shows a living cell (at time of fixation) with mitochondrial membranes clearly defined. There are (again) cytoplasmic strands in the upper part of the curl, and OPD and lipid droplets are present. A slime-like substance surrounds the hair cell.

Serial sections through the hair determined that the point of invasion was not within the crease of the curl, but at the side of it, probably at a contact point between this hair and the uninfected hair cell against it (compare Figs. 11.13 and 11.14). The point of invasion is shown in Fig. 11.14. If there had been a microcolony facilitating the
invasion, it was not detected. It is possible that a single bacterium penetrated, as described in 11.3.1, and being successful, was able to divide quickly within the forming infection thread(s). Though there is a concentration of OPD around the infection threads (Figs. 11.13-11.14) they are present throughout the cell (Fig. 11.16). Blister protrusions of the plasmalemma at the cell wall contain OPD and also occur throughout the infected cell (Figs. 11.16-11.18). An interesting aspect of this particular specimen was the orientation of microfibrillar structures in the cell wall at the pivot of the curl. The microfibrils orient vertically in the wall around the pivot, but are parallel to the plane of the wall away from this region (Fig. 11.15, see discussion on this topic in Chapter 4).

11.3.4 MICROTUBULES

A transverse section of another mature hair infection (Figs. 11.16-11.18) through a large area of cytoplasm revealed a pattern of microtubules never observed with strain ANU240 infection. The function of microtubules in this area is difficult to determine, their normal function and position being associated with the cell wall. However, it is possible that they are involved in the direction of synthesis of the infection thread, or even in the direction of OPD against the threads.

11.3.5 BACTERIAL PROCESSES

An additional peculiarity to the strain ANU2861 mutant is the formation of a process within the bacterial cytoplasm (Fig. 11.19). It can be described as a circular electron dense body (circular in cross-section - possibly a spherical body), in diameter approximately one
sixth the length of the bacterium, and surrounded by overlapping vesicles, each about one quarter the size of the large body. In sum, its shape in cross-section is similar to the flowering head of an *Helianthus*. The process is unknown by anyone in this Research School and cannot be found in any recent publication of the electron microscopy of bacteria (e.g., Iterson 1984).

11.3.6 INHIBITION EXPERIMENT

In a variety of host-pathogen situations, stimulation of plant resistance by avirulent strains can cause increased resistance against virulent strains (Sequeira et al. 1977). As the mutant strain ANU2861 in all aspects appears like an avirulent strain, an experiment designed to test for increased resistance against the wild-type by the plant was carried out. Plants were first inoculated with strain ANU2861 followed by the wild-type strain ANU240 at various intervals (Table 11.1). Although a low mean nodule per plant at 0 hr may reflect competition for infection sites between strain ANU2861 and strain ANU240, no reduction of nodulation by pre-inoculation with strain ANU2861 occurred. It is concluded that the host reaction substances stimulated by strain ANU2861 are either at insufficient levels or are compounds which cannot affect the wild-type strain ANU240.

11.4 DISCUSSION

11.4.1 NEAR-SURFACE CURLING IN THE INFECTIBLE ZONE

Ultrastructural observations of the hair curling caused by strain ANU2861 have shown that the mutant elicits a rejection response from the plant, similar to a hypersensitive reaction (Tomiyama 1983). As
the bacteria penetrates the wall of the curled hair, osmiophilic droplets (OPD) appear in the region of the penetration and at low concentration in the cell generally. These OPD are possibly phenolic compounds or phytoalexins commonly induced in pathogenic/plant relationships and most probably caused the failure of the infection (Ingram et al. 1976; Miller and Maxwell 1984). In addition, any host cell-bacterial cell communication necessary for continued infection may be precluded by the distance of the nucleus from the infection site. It is difficult to say whether the nucleus has moved away from the infection site because of incorrect 'signals' by the rhizobia, or whether the nucleus was not stimulated to move to the site because of non-existent, ineffectual or insufficient 'signals'.

These essentially 'pathogenic responses' by the plant support the hypothesis that the Rhizobium-legume symbiosis is a 'controlled disease', strain ANU2861 in this case perhaps having lost a controlling factor when it penetrates the hair wall, though it is equally possible that strain ANU2861 may actually induce the response. Thus, contact with the plasmalemma may be yet another stage for host-specificity. Another possibility of infection failure may be connected with an effect of the adenine deficiency in this mutant. It has been suggested that the failure of infection, which occurs at a position next to the plasma membrane, is the result of a failure of 'communication' between host and bacteria. It is known that adenine affects the excitability of plant cells and their ability to generate electric impulses (Vyskrebentseva et al. 1970), as well as potassium transport in plant roots (Vakhimstrov & Listova 1967). It has also been hypothesized that adenine takes part in the regulation of the value of the membrane potential of root hairs (Krasavina & Ktitovora
1975). It is feasible therefore, that *Rhizobium* strain ANU240, during the course of infection, is able to alter the membrane potential of root hairs when it reaches the plasma membrane, using adenine or adenine derivatives, and as a consequence is able to promote or set up an interaction with host molecules. Strain ANU2861, on the other hand, being deficient in the production of adenine, is unable to 'communicate' with the host in such a way, and infection fails as a result. Exogenously supplied adenine probably fails to assist infection because it is of the wrong form to promote infection.

11.4.2 CELL DIVISION

Stimulation of cortical cell division, induced by ANU2861 without any obvious indication of entry into a root hair and with no infection thread formation, suggests that the bacterial signals responsible can be transmitted into the root cortex without invasion. There were OPD here, and necrosis of sub-epidermal cells suggests a response similar to the hypersensitive reaction (Doke et al. 1982; Klement 1982; Tomiyama 1982), although such necrosis may be the result of crushing pressure from cortical cell division. Bauer's group (Calvert et al. 1984) have found that cell divisions in soybean can be stimulated without infection under normal inoculation conditions.

11.4.3 MATURE HAIR INFECTION

Of the hundreds of plants studied during the thesis work, mature hair infection was never found to occur when inoculated with strain ANU240. That such infection occurs with the mutant strain ANU2861 may merely represent the opportune infection of hairs normally inhibited to infection by the large number of successful infections in the
transient zone (thus, clover infection of mature hairs may occur because of a lack of this inhibition). In addition, infection was not found below the region of nodules, aborted infections were found only in the region of nodulation. Calvert *et al.* (1984) also found infections in soybean in these areas only. Infection threads are formed in the mature hair infection, causing a dilemma in interpretation in light of the observations on near-surface (or transient zone) curls. If the surface curls are unable to cause infection thread formation due to an inability to proceed further than the plasmalemma/wall interface, why should infection proceed further in mature hairs? A possible answer may be the position of the nucleus at the time of penetration. It is equally possible that there is a delay in recognition and response by the plant. The answers to such questions will require further investigation. Although the mature infection fails, due most likely to a host reaction indicated by the presence of OPD, its infection by strain ANU2861 may indicate differences in the physiological components between mature and emerging hairs. This may explain the existence of the transient zone of susceptibility - the reason emerging hairs are the most susceptible to infection. Certainly it is known that various compounds are transient in their presence during trichoblast formation and subsequent hair emergence (Dosier and Riopel 1977). Thus, emerging hairs, being highly sensitive to infection, may also be more sensitive to incorrect levels of elicitors and signalling compounds from the rhizobia. Mature hairs are possibly less sensitive to these levels or require smaller amounts due to the increased levels of unknown compounds relative to the levels of those compounds in emerging hairs.
TABLE 11.1 Strain ANU2861 followed by strain ANU240 at various intervals afterwards. Nodule numbers per plant, observed at 11 days after inoculation.

<table>
<thead>
<tr>
<th>Time (hrs)</th>
<th>Number of plants</th>
<th>Mean number of nodules</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>12</td>
<td>4.5</td>
<td>2.07</td>
</tr>
<tr>
<td>4</td>
<td>12</td>
<td>6.9</td>
<td>2.35</td>
</tr>
<tr>
<td>13.5</td>
<td>10</td>
<td>6.3</td>
<td>2.87</td>
</tr>
<tr>
<td>24</td>
<td>12</td>
<td>5.5</td>
<td>2.78</td>
</tr>
<tr>
<td>48</td>
<td>12</td>
<td>5.0</td>
<td>2.73</td>
</tr>
<tr>
<td>ANU240</td>
<td>11</td>
<td>5.6</td>
<td>2.42</td>
</tr>
<tr>
<td>ANU2861</td>
<td>12</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Treatments were: 0 hours, strain ANU2861 + strain ANU240 mixed culture of equivalent growth phase and density; 4 hours, strain ANU240 added after 4hr incubation with strain ANU2861; further delays of strain ANU240 inoculation for 13.5, 24 and 48 hrs. Controls: strain ANU240, strain ANU2861, uninoculated plants.
CHAPTER 12

MUTANT STUDIES 4: STRAINS ANU845(pRt032), ANU289(pJB5J1) AND ANU1260

well known, but few studies have been done on their nodules anatomy (e.g. Newman et al. 1977; van der Meer et al. 1984; Wiberg 1982; Kuykendall et al. 1988). Most of the studies, some have examined a high number of legumes, and only one (Mira et al. 1983) has attempted to correlate known genes with the phenotype. Using RFLP analysis in the Nod region of the 5m plasmid, the ineffective strains in the other studies were spontaneously generated mutants.

Little is known of the effects that different axillary backgrounds have on nodulation development. In this Chapter, the effects of changing when the nod "flip" mutants on temperate (clover) and tropical (legume) plants are examined. Many temperate legumes form indeterminate nodules and most tropical legumes determinate. There are major differences between these two kinds of nodules, summarized in Fig. 12.1. The most important difference is the formation of an "outside" meristem in temperate nodules, which enables the nodule to grow into a nodulated form. At a consequence, the vascular system is quite different from the determinate nodules, and there is constant growth of infective stems and infection of the newly formed cells at the tip of the nodule. This continued infection may not occur in tropical legume nodules. Thus, there appears to be two phases of meristematic activity in nodules. Firstly, the stimulation of root cortical and pericentral cells to form the initial round compact of the early nodule followed by enlargement of those cells; and secondly, the formation of an "outside" meristem in temperate nodules only where continuous
12.1 INTRODUCTION

Rhizobium mutants that cause an ineffective (Nod$^+$ Fix$^-$) phenotype are well known, but few studies have been done on their nodule anatomy (eg Newcomb et al. (1977) - pea; Werner et al. (1980) - soybean; Vance et al. (1980) - alfalfa; Bassett et al. (1977) - soybean; Hirsch et al. (1983) - alfalfa). Of these studies, none have examined a tropical legume, and only one (Hirsch et al. 1983) has attempted to correlate known genes with the phenotype (using Tn5 insertions of the nif region of the Sym plasmid), the ineffective strains in the other studies were spontaneously generated mutants.

Little is known of the effects that different genetic backgrounds have on nodule development. In this Chapter, the effects of three kinds of Nod$^+$ Fix$^-$ mutants on temperate (clovers) and tropical (siratro) plants are examined. Many temperate legumes form indeterminate nodules and most tropical legumes determinate. There are major differences between these two kinds of nodules, summarized in Fig. 12.1. The most important difference is the formation of an 'extending' meristem in temperate nodules, which enables the nodule to grow into a cylindrical form. As a consequence, the vascular system is quite different from the determinate nodule, and there is constant growth of infection threads and infection of the newly formed cells at the tip of the nodule. This continuous infection does not occur in tropical legume nodules. Thus, there appears to be two stages of meristematic activity in nodules. Firstly, the stimulation of root cortical and pericyclic cells to form the initial rounded form of the early nodule, followed by enlargement of those cells; and secondly, the formation of an 'extending' meristem (in temperate nodules only) where continual
division provides cells for infection by the microsymbiont (see Newcomb 1981). In the initial stages, there appears to be little difference morphologically between tropical and temperate legume nodules.

There have been many studies on infection and nodule development (see review by Dart (1977), but little has been done to bridge the gap between cytology and molecular biology. By using specific kinds of mutants and constructed strains, the underlying features and mechanisms of infection and nodule development can be probed.

12.2 MATERIALS AND METHODS

12.2.1 BACTERIAL STRAINS

Strain ANU843 (Rolfe et al. 1980a) is an R. trifolii strain that nodulates and fixes nitrogen on white and subterranean clover.

Strain ANU845 is a Sym-plasmid cured derivative of strain ANU843 which is unable to infect and induce nodules on clover roots (Hac− phenotype).

Strain ANU845(pRt032) is strain ANU845 carrying the 14.0kb HindIII DNA fragment which has the nodulation region of R. trifolii (Schofield et al. 1984)(Fig. 12.2).

Strain ANU289 is a nonmucoid (on mannitol medium) Smr− derivative of the Bradyrhizobium strain CP283, isolated from the tropical elm Parasponia by Trinick and Galbraith (1980) that nodulates and fixes nitrogen on siratro.

Strain ANU289(pJB5J1) carries the R. leguminosarum Sym plasmid pJB5J1, which is a 130Mdal plasmid carrying the genes for the Nod, Nif and Hsp phenotypes on peas. This tropical strain is unable to form nodules on
peas or subterranean clover, but will still nodulate siratro, though these nodules have a Fix\textsuperscript{-} phenotype.

Strain ANU1260 is a nodulation-defective derivative of strain ANU240 carrying a single Tn5 in the chromosome.

12.2.2 PLANT ASSAY

Purified bacterial colonies were inoculated onto nitrogen-free medium for rapid plate plant assay (Rolfe et al. 1980b) with seedlings of *Trifolium repens* (New Zealand white clover) and *T. subterranean* (Mt. Barker variety) for strain ANU843 and strain ANU845(pRt032); or with siratro seedlings for strain ANU289, strain ANU289(pJB5J1) and strain ANU1260.

12.2.3 MICROSCOPY

The most mature (largest) nodules from 10 plants per treatment were excised and fixed at 9, 12, 15 and 18 days after inoculation for white and subterranean clover. Nodules of varying sizes at 21 days for strain ANU289, strain ANU289(pJB5J1), and strain ANU1260, were excised and fixed. Fixation and embedding followed the standard schedules set out in Chapter 2.

12.3 THE TRANSCONJUGANT STRAIN ANU845(pRt032)

This study was done in conjunction with Mr. Peter R. Schofield (Genetics Department, R.S.B.S), who supplied the mutant Rhizobium and the nodule material. My contribution involved all the microscopy work.
The methods of mutagenesis and isolation of the mutants is described in a paper published on this work (Schofield et al. 1984) and this Chapter will discuss the detailed microscopic evidence, necessarily omitted from the paper. Description will concentrate on nodule and bacteroid development rather than the infection process. The wild type infection of clover is well documented (see Fahraeus 1957; Dart 1974, 1977). A brief summary of the published work will put this Chapter in context.

The Rhizobium trifolii genes necessary for nodule induction and development have been isolated on a 14.0kb HindIII fragment of the Sym plasmid DNA (Fig. 12.2). When cloned into a broad-host-range plasmid vector, these sequences confer a clover nodulation phenotype on a derivative of R. trifolii which has been cured of its endogenous Sym plasmid. Furthermore, these sequences encode both host specificity and nodulation functions since they confer the ability to recognise and nodulate clover plants when they are transferred to strains of Agrobacterium tumefaciens and strain ANU240. This indicates that the bacterial genes essential for the initial, highly-specific interaction with plants are closely linked (Schofield et al. 1984).

12.3.1 WHITE CLOVER NODULATION: STRAIN ANU843 - WILD-TYPE CONTROL
Colonization and curling (Figs. 12.3, 12.4) occurred within 24 hours. Earliest nodules appeared within three days and were easily distinguished by the red pigment anthocyanin in the outer part of the nodule.

12.3.1.1 NINE DAYS AFTER INOCULATION
By 9d after inoculation, nodules were seen as small bumps on the root surface of about twice the diameter of the root (Fig. 12.11A), and
nodule cells are extensively invaded by infection threads. Host cells that contained released bacteria were highly vacuolate (Fig. 12.5A) with a centrally located nucleus. The *Rhizobium* were found to be 'packaged' in peribacteroid membranes, numbering 1-8 per package (Fig. 12.5B). Some *Rhizobium* cells were swollen and had become pleomorphic. Each *Rhizobium* cell contained large crystals of polyhydroxybutyrate (PHB) (Dart 1977). The cytoplasm was rich in mitochondria, rough endoplasmic reticulum (RER) and dictyosomes, reflecting the highly active state of the cell at this time.

12.3.1.2 TWELVE DAYS AFTER INOCULATION

By 12d, nodules were larger and had a greater number of bacteria-filled cells. The cells were no longer vacuolate but the nucleus remained in a central position (Fig. 12.6). Quite well-formed bacteroids were contained singly in peribacteroid membranes. There were still a number of multiply-packaged *Rhizobium*, which are presumed to be those partitioned off most recently from the infection threads. Infection threads were now less obvious amongst the differentiating bacteroids. The well-formed bacteroids contained little or no PHB. Starch granules were now present at the periphery of each bacteroid-containing host cell. The cell cytoplasm still appeared to be dense and active.

12.3.1.3 EIGHTEEN DAYS AFTER INOCULATION

The 18d sample (Figs. 12.7-12.9) showed development of the central vacuole typical of mature clover infected nodule cells (Dart 1977). The nucleus was placed next to this vacuole. There were no undeveloped packages of *Rhizobium*, as they had all developed into bacteroids. There was a little PHB in some cells. All bacteroids were packaged singly in peribacteroid membranes. There appeared to be
a diminished number of mitochondria and dictyosomes but RER was still very prominent in the cell cytoplasm. A small number of electron-dense Rhizobium were present in each cell and probably represent unsuccessful bacteroid formation, perhaps the result of membrane rupture. The nodule was now an extended shape (approximately 2mm long) having developed an 'extending' meristem which produces the elongated morphology of the indeterminate clover nodule (Fig. 12.8).

12.3.2 WHITE CLOVER NODULATION: STRAIN ANU845(pRt032)

Macroscopically, nodules grew in a similar manner to strain ANU843 nodules for the first 12d. However, no extending meristematic zone developed, and the nodule remained essentially unchanged after 12d (Figs. 12.10B and 12.11). There were generally twice the number of nodules compared to the wild type noduleation (P.R.Schofield pers. comm.). The most immediate impression at the LM level of observation is the lack of formation of bacteroid-containing cells (Fig. 12.10B). A 9d sample at TEM level (Fig. 12.12A,B) showed PHB-filled Rhizobium were released in large clusters into highly vacuolated cells. In Fig. 12.12C, a high magnification of a 12d sample, the Rhizobium appear to have been released into the cell as a large 'droplet'. Some Rhizobium cells were enlarged and become pleomorphic, others seemed to be plasmolysed. A 15d sample was quite similar but the Rhizobium were more dispersed in the cell. There was more membrane material and less Rhizobium evident, of which fewer were bacteroid-shaped. By 18d, large amounts of membrane material had accumulated in some plant cells (Fig. 12.13). No bacteroid-like Rhizobium were present. Most Rhizobium had little or no PHB. There was little cytoplasm in the host cells that had bacteria released in them. The nodule size
increased only marginally from 12 to 18d and remained spherical in shape because of the lack of development of an extending meristem. Infection threads appeared quite prominently because of the lack of proper infection; wild-type infection threads are more difficult to discern because of the numbers of bacteroids.
12.3.3 SUBTERRANEAN CLOVER NODULATION: STRAIN ANU843 - WILD TYPE

CONTROL

Subterranean clover nodules developed more quickly than those on white clover, and 9d samples (Fig. 12.14) showed well-developed bacteroids packaged singly in peribacteroid membranes. Some of the smaller bacteria, however, were still multiply-packaged (Fig. 12.14B). The host cell nucleus was prominent and centrally located, there were small vacuoles throughout the cell and small amounts of peripheral starch granules were present. Host cell cytoplasm was dense and active. By 18d (Fig. 12.15) the bacteroids were all well developed and packaged singly. There were still a number of small vacuoles amongst the bacteroids, but no large central vacuole had developed. Peripheral starch granules had developed further (Fig. 12.15A). No PHB was present in the bacteroids at all sample points. Ferritin-like crystal formation was common (Fig. 12.15C). The number of mitochondria and dictyosomes was considerably decreased by 18d but RER was still prominent.

12.3.4 SUBTERRANEAN CLOVER NODULATION: STRAIN ANU845(pRt032)

Nodules failed to develop meristems and subterranean clover nodules remained globose, essentially unchanged from 12d. By 18d host cells into which Rhizobium had been released had large amounts of peripheral starch (Fig. 12.16). Host cell cytoplasm had larger numbers of mitochondria and dictyosomes compared to equivalently-aged cells in the strain ANU843 control. Bacteria were mostly multiply-packaged and small. Those bacteria that had enlarged to bacteroid shape were generally half the size of strain ANU843 bacteroids. Dense bacteria that appeared to be degenerate were present (Fig. 12.16B).
12.4 THE TRANSCONJUGANT STRAIN ANU289(pJB5J1)

In a series of experiments conducted by Rolfe et al. (1983), the pea Sym plasmid pJB5J1 was transferred into a number of fast and slow growing Rhizobium strains, with variable expression of the plasmid-borne symbiotic functions. One of these slow-growing transconjugants, strain ANU289(pJB5J1), showed no delay in the onset and early development of nodulation, when infecting siratro, but the nodules were ineffective. This transconjugant was examined in detail and the results are presented here.

12.4.1 STRAIN ANU289 - WILD-TYPE CONTROL

Infection by strain ANU289 of siratro produces determinate nodules (Fig. 12.17). The bacteroids developed in the nodule (Figs. 12.18-12.20) were small compared to those in clover, enlarging only about 30% of bacteria size. In addition, mature bacteroids were multiply-packaged in a peribacteroid membrane, not singly, as in clover. These three factors, nodule shape and size and packaging of bacteroids, are major distinguishing features between temperate and tropical legume nodule anatomy. Other distinguishing factors are position of vascular tissue, extent of infection thread ramification, presence of transfer cells, presence of lenticels and sclereids in the outer cortex (see Dart 1977).

12.4.2 STRAIN ANU289(pJB5J1)

The transconjugant produced small, ineffective (Fix-) nodules. Examination of the nodule interior showed no development of bacteroid-containing tissue (Fig. 12.21). Higher magnification showed release
of Rhizobium 'naked' into the host cell, where they were apparently lysed and degenerated (Fig. 12.22). Almost no bacteroid formation was observed and generally no peribacteroid membrane synthesis was seen in any host cell (Fig. 12.21). The host cells still had active cytoplasm and host cell nuclei were often observed near infection threads.

12.5 THE MUTANT STRAIN ANU1260

Amongst the large number of mutants of strain ANU240 produced by Morrison (1984) and Mr. Ying Hua Cen (Genetics Department, R.S.B.S), one interesting Nod^+ Fix^- mutant was examined in detail. The wild-type nodulation of the parent strain ANU240 is, in all appearances, exactly the same as the control strain ANU289 above (Figs. 12.17-12.20).

Strain ANU1260 nodules were small, slow to develop, and ineffective (Fig. 12.23) and contained large amounts of starch (Figs. 12.23, 12.24). Bacterial release was confined to small groups of host cells in the nodule centre (Fig. 12.24B,C). Higher magnification of those cells at TEM shows that bacteria were released into membranes and that a few changed into a bacteroid form (Fig. 12.25). The host cytoplasm was degenerated, but RER and mitochondria were present (Fig. 12.25B,C). The Rhizobium were filled with PHB. Amyloplasts (Fig. 12.26) were common throughout the nodule tissue, and were the organelles responsible for the starch accumulation. Although membranes surrounded the Rhizobium, there was considerable amounts of membrane material free in the host cell (Fig. 12.26).
Normal nodule development induced by fast-growing *Rhizobium* strains such as *R. trifolii* leads to an indeterminate cylindrical shape (Figs. 12.1 and 12.8). The genetic information encoded on plasmid pRt032, however, is insufficient to allow proper nodule formation, as an 'extending' meristem does not develop, and the nodules remain globose. During the formation of white clover nodules infected by strain ANU843, *Rhizobium* are released from the infection threads into a peribacteroid membrane synthesized by the host. The *Rhizobium* subsequently undergo division within this membrane and become individually packaged as they swell into the bacteroid form. With the transconjugant strain ANU845(pRt032) however, *Rhizobium* release appears to be uncoordinated with membrane synthesis and *Rhizobium* appear to be released directly into the host cytoplasm, whilst membrane synthesis ensues elsewhere in the same cell (Figs. 12.10-12.13). This result is very similar to that for an ineffective mutant of pea reported by Newcomb et al. (1977). They demonstrated that bacteria released from the bacterial thread via an unwalled droplet were not always surrounded by a host membrane. In later stages of nodule development they found many infected cells containing rhizobia with no enclosing membranes. Thus, the biosynthetic capacity of the cells appeared to be impaired and membrane synthesis defective. The authors suggested that failure of the nodules to develop nitrogenase activity was probably related to the failure of membrane formation around the bacteria. In the case of strain ANU845(pRt032), however, nitrogenase activity could not develop because the nif region is missing. Thus, it is possible that some of the nif genes are involved
in the direction of membrane synthesis, although the two \texttt{nif::Tn5} \texttt{(nifH\textsuperscript{-}, nifDK\textsuperscript{-})} mutants of Hirsch et al. (1983) developed normal bacteroid structures. Nevertheless, the strain ANU845(pRt032) is still capable of bacteroid formation. If genes are involved in bacteroid formation then they are either on the pRt032 insert fragment or on the bacterial chromosome. Plasmolyzed bacteria reflect the lysing action of the host cytoplasm when no protective membrane is present. The bacteria are probably recognised as true 'invaders'. Lack of cytoplasm and apparent death of the host cell by 18d is a typical host response to pathogenic invasion (Tomiyama 1983).

When infected with strain ANU843, subterranean clover developed singly-packaged bacteroids in a similar manner to white clover, though at a faster rate. Unlike white clover, strain ANU845(pRt032)-infected subterranean clover plants showed coordination of membrane and \texttt{Rhizobium} release, some becoming enlarged and singly packaged, though at half the size of equivalent wild-type bacteroids. Others remained small and multiply-packaged or appeared to degenerate (Fig. 12.16B).

In all three examples presented above, breakdown of the symbiosis occurs at either the moment of bacterial release, or soon after. In the first presented situation, strain ANU845(pRt032) on white clover, bacterial release and peribacteroid membrane synthesis seemed to be uncoordinated, whereas on subterranean clover there was coordination. This points to host specificity differences. However, there may be cotyledon nitrate effects because of the size differences between the small-seeded white clover and subterranean clover, which has a comparatively large seed. How these reserves of nitrate may affect the rate of infection and, as a consequence, the development of the
infection process is unknown and should be investigated before final conclusions are drawn about host specificity. On the other hand, if nitrogen fixation doesn't start until the formation of bacteroids, then it should be assumed that normally there is enough cotyledonous nitrogen in white clover to form bacteroids before any deleterious effects of a lack of such nitrogen will show. Thus, the absence of normal bacteroid formation by this transconjugant may be a real effect.

The second strain, ANU289(pJB5J1), releases bacteria directly into the host cytoplasm, as strain ANU845(pRt032) on white clover, but is unable even to stimulate membrane synthesis.

The third mutant, strain ANU1260, has limited success in packaging released bacteria, but uncoordinated membrane synthesis does occur. This strain is very interesting in light of the results of Vance et al. (1980) who studied an alfalfa strain (MnPL480) which produced Nod⁺ Fix⁻ phenotypes when infected by normally effective R. meliloti strains. These authors described tumour-like nodules filled with starch and containing few infected cells and little proliferation of infection threads. The infected cells rapidly senesced. These results are very similar to the effects of strain ANU1260, and it is tempting to speculate that strain ANU1260 is unable to induce a plant gene involved in nodule synthesis.

In all cases, to greater or lesser extents, bacteria are seen changing into bacteroid forms, though none succeed in developing into normal bacteroids (see diagrammatic summary of results, Fig. 12.27). These results suggest that certain infection stages are under at least partial direction of the bacterial genome. Enough samples of each mutant were examined to preclude any host variation.
All mutants showed partial bacteroid formation, demonstrating that at least initiation of bacteroid formation is under bacterial control. The gene products necessary for this may need containment in peribacteroid membrane, exemplified in strain ANU845(pRt032)-infection of subterranean clover. However, as these subterranean clover mutant bacteroids reach only approximately 50% of control size and are not singly packaged, it is possible that other genes or host products are required for bacteroid maturity. It is also possible that bacteroid differentiation is non-genetically controlled, for bacteroid-like Rhizobium can be grown on media with high concentrations of sugar. Bacterial release occurs with all three mutants, regardless of membrane synthesis, and is thus most likely to be under bacterial control.

Membrane synthesis is presumably stimulated by Rhizobium genes. In the case of strain ANU289(pJB5J1), the introduced plasmid caused a disruption of the normal plant membrane synthesis. If membrane synthesis was totally host-controlled, it would be expected to either occur or not, in the three bacterial infections. The phenotypic differences of the three mutants described may be due to host differences. However, the fundamental principles of bacterial release and the concurrent membrane synthesis are highly conserved in bacteroid-forming legumes (Dart 1977), and it may be argued that all legumes share genes which control basic functions in Rhizobium (Fig. 12.27). More mutants are needed that, for example, release Rhizobium in clover without membrane synthesis, to be sure that such genes are conserved.

For clovers, the results suggest a number of possible controlling factors:
1. The plant may be capable of detecting nodules infected by the transconjugant as ineffective, and aborts (by non-development of the 'extending' meristem) at an early stage (see Nutman 1952). Work by Bohlool's and Bauer's groups (P. Gresshoff, pers. comm.) shows that autoregulation starts prior to the onset of fixation, and is a recognition by the plant of meristematic activity. This can be a positive or negative mechanism, the plant either produces only so much of a key substance needed to support meristems, or meristems produce an inhibitory substance which prevents other meristems from developing.

2. It is possible that missing genes, perhaps those in the nif region (Scott et al. 1981; 1982) are required to transmit the correct 'messages' to the host.

3. Kondorosi et al. (1982) suggested that expression of genes involved in the later stages of the symbiosis may be host-specifically regulated. The results presented here are consistent with these ideas. The contrasting developments of strain ANU845(pRt032) in white and subterranean clover point to host specificity differences. However, nitrate controls for these experiments were not performed and should be done before more specific conclusions can be made.

Overall, each strain produces nodules that appear to senesce immediately, and it seems clear that the strains are unable to cope with the host cell environment, even when only a single gene has been interrupted (as in the case of ANU1260).

Studies of these kind suffer from an inability to locate infection at a very early stage, and serve to emphasize the need to localize
infection both spatially and temporally. Such studies, known to be possible in soybean (Turgeon and Bauer 1982) and siratro (this thesis - see Chapters 4-6) will assist the examination of the earliest stages in infection thread ramification and bacterial release.

The use of the Sym plasmid-cured bacterial background (eg strain ANU845) to assay the functions encoded on subfragments of the Sym plasmid is valuable for the isolation and analysis of host-specific nodulation genes (Buchanan-Wollaston et al. 1980; Banfalvi et al. 1981; Kondorosi et al. 1982; Schofield et al. 1984). Further development of this system should allow more precise definition of the nodulation process itself and the functions encoded by the various genes involved.
CHAPTER 13

GENERAL DISCUSSION

Synthetic nitrogen fixation is of substantial importance to the world economy. If efforts to improve the use of legumes are to be successful, it is paramount that we understand legume-bacteria biology, especially at the very earliest stages of infection. Ideas such as the role of specific factors in legume infection, such as Rhizobium vectors, are crucial. Improving our competitiveness characteristics in inoculate already improved nitrogen fixation, or changing the regulation map on the legume, is expected to a greater or lesser extent on the nucleotide level of the first and early interactions between the legume (or non-legume) root and Rhizobium.

It is testimony to the difficulties involved in studying bacteria-plant interactions, such as the Rhizobium-legume system, that until recently, little has been known of the earliest stages of infection. The Rhizobium-legume symbiosis is one of the most difficult to study because of the very low frequency of infection that generally occurs on a given root. The turning point came when Shewmaker et al. (1966) reported that inoculated root cells have a transient susceptibility to infection. Taking advantage of this, Towers et al. (1986, 1987) used a micro-isolation technique that allowed them to infect only the most apoplastic cells and thus more precisely and reliably analyze the early stages of infection of soybean. Their studies, however, were hampered mainly at low magnification, and they published few TEM micrographs.

The studies presented in this thesis have taken the analysis of infection one step further by examining the sequence of infection events in more detail at high magnification and using mutants and transconjugants. Bacterial strains as probes to characterize the bacterial genome, thereby revealing previously unknown aspects of
Symbiotic nitrogen fixation is of fundamental importance to the world economy. If efforts to improve the use of legumes are to be successful, it is paramount that more be known of Rhizobium-legume biology, especially at the very earliest stages of infection. Ideas such as broadening the host range of Rhizobium (ie changing host specificity), improving soil competition characteristics of rhizobia already improved in nitrogen fixation, or changing the nodulation rate on the legume, all depend to a greater or lesser extent on how much is known of the first and early interactions between the legume (or non-legume) root and Rhizobium.

It is testimony to the difficulties involved in studying bacterial-plant interactions, such as the Rhizobium-legume symbiosis, that until recently, so little has been known of the earliest stages of infection. The Rhizobium-legume symbiosis has been particularly difficult to study because of the very few successful infections that generally occur on a given root. The turning point came when Bhuvaneswari et al. (1980) reported that soybean root cells have a transient susceptibility to infection. Taking advantage of this, Turgeon and Bauer (1982, 1983) devised a micro-inoculation technique that allowed them to infect only the most susceptible cells, and hence precisely and reliably analyse the early stages of infection of soybean. Their studies, however, were conducted mainly at low magnification, and they published few TEM micrographs.

The studies presented in this thesis have taken the analysis of infection one step further by examining the sequence of infection events in more detail at high magnification, and using mutant and transconjugant bacterial strains as probes to characterise the bacterial genome, thereby revealing previously unknown aspects of
infection. These studies were mostly carried out on the tropical legume siratro, which is infected via the root hair and infection threads. No publication exists on the detailed analysis of infection of a tropical legume infected via root hairs.

In many respects, it appears that the Rhizobium-legume symbiosis parallels events in plant pathogenesis. One mutant in particular in these studies (ANU2861) has demonstrated that Rhizobium-legume infection bears striking resemblance to infection and symptom expression induced by plant pathogenic organisms.

Before examining the Rhizobium-legume symbiosis in a phytopathogenic perspective, a summary of the infection events, based mainly on the strain ANU240-siratro symbiosis will be presented and discussed in terms of the phenotypic code, first devised by Vincent (1980) and Rolfe et al. (1981)(see Table 1.1).

13.1 SUMMARY OF INFECTION EVENTS IN THE SYMBIOSIS IN RELATION TO THE PHENOTYPIC CODE.

1. Root colonization (Roc). Strain ANU240 appears to colonize the siratro root surface in a manner similar to other rhizobia in other systems. However, if the Sym plasmid is lost (by heat-curing) from the strain ANU240 (strain ANU265) then colonization around a micro-inoculated area appears to be more limited. The same occurs with the Hac⁻ Nod⁻ strain ANU1255, which has only a single Tn5 in the hair curling region (nodD) of the Sym plasmid. In both cases, there is a complete inability to curl hairs and infect. In addition, strain ANU240 causes erosion holes in the surface of the root, a phenomenon that was not seen in
strains ANU265 and ANU1255. Thus, infection at least, and perhaps even the ability to colonise the root surface, can be disrupted by mutation of a single gene. This result points to the importance of a switching concept, first introduced by Heath (1974) in the context of pathogenic organisms, where each stage in infection is controlled by a single gene which can switch 'on or off', affecting the subsequent events of infection (Fig. 13.1). Thus, in the case of mutant strain ANU1255, the disruption of the nod D gene has led to the non-expression of other bacterial genes involved in infection.

2. Root adhesion (Roa). Adhesion (or attachment) to the root surface appears to be general, although in some observations at SEM and FM rhizobia were concentrated along the edges of epidermal cells. These parts of the surface tend to retain root cap slime (as several lectins demonstrated) and it is possible that the rhizobia are attracted to the epidermal edges by the sugars of the root cap slime, perhaps in particular B-D-Galactose, which is also expressed at root hair tips.

3. Root hair attachment. Attachment of the lectin RCA I at the siratro root hair tip suggests that the sugar to which it binds (B-D-Galactose) may be involved in the recognition event that takes place there. It is clear from the nature of curled hairs that Rhizobium-mediated curling occurs as the hair emerges from the trichoblast. The sugar can be detected on the surface of the trichoblast even before the hair can be observed by any other light microscopic technique used in this study. It is thus
possible that B-D-Galactose is involved in the recognition and attachment of the Rhizobium to this highly-localised part of the trichoblast surface. When the root surface was first incubated with RCA I lectin, infection and nodulation by strain ANU240 were prevented. It is possible that, because of the highly specific nature of sugar recognition (compare for example the single sugar differences between A and O blood groups), the event of attachment at this site is the first host specific event. If this is the case, then the strain ANU1255, which is unable to curl root hairs, may be a host specificity mutant. The localization of the B-D-Galactose sugar at the hair tip most likely acts as a target for Rhizobium. When the Rhizobium cell reaches the emerging hair, genes that code for the enzymes that cause perturbation of normal hair tip growth (hair curling genes) are triggered. Thus, erosion holes at the epidermal surface may represent random triggering of these hair curling genes.

4. Hair curling (Hac): expression of hair curling genes. For the hair to curl over, it is clear that Rhizobium must somehow affect the hair cell wall. Any inert object placed on a growing tip will be quickly moved to the equator of the dome hemisphere. From the nature of curled hairs, it is clear that Rhizobium is not moved to the dome equator, and therefore must affect the root hair tip growth processes at a localised spot. From observations of curled root hairs and micro-colonies of Rhizobium on the surface of root hairs, it appears that the bacteria are able to prevent normal microfibril polymerization, and fibrils that would normally orient parallel to the wall surface, instead, are oriented perpendicular
to it. Thus, it is possible that the rhizobia secrete enzymes that preclude normal polymerization processes. This localised spot of perpendicular microfibrils would be inherently weak compared to the rest of the hair cell wall. As the rhizobia have affected growth process at the tip and not reached the dome equator, the tip grows asymmetrically, pivoting at the weak spot, and forming the curl. When the hair tip reaches the epidermal surface it often continues to grow along the surface of the root for a short distance. This growth is not asymmetric and it would appear that normal tip growth processes are restored. This restoration would most likely occur as the pivoting tip meets the hair surface below the pivot, and the tip is prevented from further curling. In such cases where the curl does not move directly towards the root surface but at an angle to it, the tip will miss the root surface and continue to curl, creating a 'corkscrew'.

5. Colonization of the hair enclosure. Rhizobia multiply in the enclosed pocket created by the curled hair. As the Rhizobium is normally unable to infect hairs without curling, it is suggested that the enclosure allows for the accumulation of wall-degrading enzymes that would normally leak away from an exposed colony at the root surface. It is also quite feasible that, after penetrating the hair cell wall, the curl acts as a pressure seal against turgor pressure. (NB There is some evidence to suggest that occasionally an infection can occur between two overlapping hairs, which nevertheless create an enclosed area, see Bauer 1981).
6. Penetration of the hair cell wall. From observations of the penetration of the hair cell by *Rhizobium*, it is clear that enzymic degradation of the hair wall matrix occurs, and a loosening of the wall microfibrils allows the rhizobia to enter an interfacial zone, pushing against turgor pressure by active bacterial division. The rhizobia do not enter the cell at the weak pivot site, but at a site between the pivot and the tip end of the pocket, and always through the wall that formed above the pivot. It is feasible, therefore, that a different enzyme (and a different gene or genes) is responsible for penetration of the rhizobia into the hair cell.

7. Communication at the plasma membrane. Observations of infection at the zone of susceptibility in *siratro*, when infected by strain ANU2861, showed that the strain was unable to proceed further than the plasma membrane of the hair cell, in fact invoking a resistance-like response from the host in the form of osmiophilic droplets that may contain phytoalexin or other antibacterial substances. It seems possible that the plasma membrane is a site of 'communication' with the host, and the strain ANU2861 is unable to properly communicate. How this form of communication may occur is open to conjecture. However, it is known in some pathogenic situations (Wheeler 1976) that the pathogen is able to alter the membrane potential of the host cell. Perhaps in the normal situation, strain ANU240 is able to alter the potential of the membrane (through hormones?), alter its permeability, and act as focus for host endogenous repair mechanisms. It is likely that this stage of meeting the plasma membrane is another host specific
step. The host cell nucleus is always close to the invading site in the normal situation, but not in strain ANU2861 infection.

8. Browning and thickening of the infected hair cell surface. Between 12 and 20 hours after inoculation (ie immediately after curling) a noticeable browning of the infected hairs makes their observation quite easy. The exact nature of the wall thickening substances is unknown. It is possible that the mechanisms involved in this phenomenon may be similar to the mechanisms involved in infection thread synthesis, and the general deposition of the outer wall may occur because penetration itself has not occurred and there is thus no focus for infection thread synthesis.

9. Infection thread synthesis (Inf). If the Rhizobium is able to continuously irritate the plasma membrane at a localised spot, then the endogenous cell wall repair mechanisms will continue to be attracted to the site, and could form an infection thread. It has been suggested (see Dart 1977) that the mechanisms involved in infection thread synthesis are re-directed tip growth capabilities. There is little evidence to support this notion in the work presented here, except that curled hairs rarely grow far along the surface, and therefore tip growth may stop in favour of infection thread synthesis. Generally, several threads are formed from one infection site, and these ramify within the hair cell for 15-20 hours before orienting towards the basal part of the hair cell, usually all at the same time, so it is common to see several growing infection thread tips going towards the base of the cell together. How the threads then penetrate the basal wall and enter
the cortical tissue is unknown. However, there is always a slight swelling or spread of the infection thread as it meets the wall (Newcomb 1981) and this may create an appropriate micro-environment for rhizobial penetration enzymes to degrade the wall matrix at that point and push through the wall. One idea is that Rhizobium wall-degrading enzymes that can be stimulated by wall fragments are suppressed within the infection thread. However, when the thread meets the cortical wall, the thread tip fuses with the plasma membrane, thus exposing the rhizobia to cell wall elicitors which stimulate enzyme expression. After penetration, the thread tip fuses with the plasma membrane of the penetrated cell, separating the rhizobia from the cortical wall, and stimulation of enzyme production ceases. As the nucleus and cytoplasm of the cortical cell is there to 'meet' the invading bacterial cells (Newcomb 1981), then the rhizobia must be capable of sending message molecules in advance of the invasion. The threads penetrate the already-dividing cortical cells (Noi) below the infection site. The rhizobia also spread by invading the intercellular spaces between the cortical cells, in this case no threads are formed (nor could they be without host cytoplasm). These intercellular bacteria then penetrate and invade cells, causing infection thread synthesis as they do so. (Note: all the rhizobia are essentially intercellular, but it is convenient to distinguish rhizobia in infection threads in host cells as intracellular).

10. Bacterial release (Bar). In both temperate and tropical legumes, rhizobia are normally released into the host cytoplasm surrounded
by a host-synthesized membrane, the peribacteroid membrane (pbm) (Robertson et al. 1984a). However, in the case of strains ANU1260, ANU845(pRt032)(on white clover) and ANU289(pJB5J1), there was a disfunction at this stage, whereby the bacteria were either released without the surrounding membrane, or early breakdown of the membrane occurred (Chapter 12). In the first two cases, membrane material was produced elsewhere in the cell. It would seem then, that proper bacterial release involves the cooperation of both host and Rhizobium genomes, and that some sort of communication is necessary to coordinate the process. The fact that pbm development appeared normal in subterranean clover infected by strain ANU845(pRt032) but abnormal on white clover implies a third host specificity step.

11. Bacteroid development (Bad). In both temperate and tropical legumes, the bacteria divide within the pbm, at the same time they swell into bacteroid forms (Dart 1977). In clovers, the bacteroids (which can be many times the size of free-living rhizobia) become individually 'packaged'. In tropical legumes, the bacteroids swell only slightly and remain multiply-packaged (Dart 1977). In the case of strains ANU1260, ANU845(pRt032)(on white clover) and ANU289(pJB5J1), a few rhizobia swelled slightly, even without a surrounding pbm, suggesting that bacteroid development is at least partially under control of the bacterial genome. In the case of strain ANU845(pRt032) on subterranean clover, bacterial release appeared to be normal but bacteroids did not develop fully and failed to be packaged individually. It is clear, therefore, that the genetic information of the nodulation
region encompassed by the 14kb HindIII fragment is sufficient only to attain bacterial release in clovers. It is likely that bacterial release is controlled partially by other bacterial gene(s) and partially by a host function.

From the above summary, changes to the phenotypic code are presented in Table 13.1. Some of these changes are ideas rather than proven facts, for example, communication at the plasma membrane (Hoc). However, it is clear that the original phenotype code lacked detail, and Table 13.1 can be considered an up-date in accordance with the results and ideas presented in this thesis. Before such results can be applied generally, a large number of infection systems need to be investigated. It is likely that such investigations will show that different Rhizobium-legume systems will share some basic mechanisms of infection, but that details may differ. Obvious examples of such differences would be between temperate and tropical legumes, or between legumes that are infected via the hair and those infected via cells at the junction of tap and lateral roots (such as peanut and stylo). Thus, a number of phenotypic code tables may need to be drawn to accommodate each infection mode.
13.2 THE RHIZOBIUM-LEGUME INTERACTION FROM A PHYTOPATHOGENIC PERSPECTIVE

13.2.1 RECOGNITION

The ability to recognise (or, more correctly, cognise) non-self is a general attribute of all organisms and is the general basis for many fundamental activities of living organisms, such as fertilization, development, differentiation, and immunological responses. Recognition is the basis of the induction of disease resistance or susceptibility, and hence compatibility and incompatibility, and the results of these, symptom expression.

Albersheim and Anderson-Prouty (1975) have proposed a scheme based on the idea that the pathogen (or symbiont) carries surface molecules which act as identifying labels. If the potential host has receptors capable of recognising these, then the defence system (or symbiotic system) can be alerted. In Chapter 8, it was shown that the lectin probe RCA I could detect the presence of a sugar (β-D-Galactose) at the root hair tip. It is possible that such a molecule is the host receptor, and that (unknown) surface molecules on the strain ANU240 surface are able to interact with it in such a way as to stimulate the symbiotic system. Certainly, pre-incubation of the root surface with the RCA I lectin prevented infection by strain ANU240, presumably by saturation of all the available receptor sites. Albersheim and Anderson-Prouty (1975) suggested that the surface factors carried by the pathogen (or endosymbiont) are glycoproteins, the specificity lying in the carbohydrate part of the molecule. The recognition receptors in the host would then be complementary proteins, held in the plasmalemma, or near to it, or in the cell wall. It would seem
that in the initial stages of attachment in the strain ANU240/siratro system at least, the configuration suggested by Albersheim and Anderson-Prouty is different or perhaps reversed, the carbohydrate receptor being in the host cell wall, and an unknown (protein or carbohydrate) receptor on the microbial surface.

13.2.2 SPECIFICITY

If the specificity of the interaction lies in such factors mentioned above, then a mutation in the pathogen or symbiont, presumably involving a change in the specificity of a glycosyltransferase (which are involved in the biosynthesis of sugars) would produce a new configuration of the surface receptors. The host would not have the receptors for this, and would be susceptible to invasion by the pathogen, but not by a symbiont. This brings the argument to an important point: how does the concept of recognition conferring resistance equate with recognition conferring symbiosis, which is more likely equated with susceptibility? Recently presented arguments (see Vanderplank 1982) suggest that specificity can be equated with pathogens which establish biotrophic interactions. Some of these pathogens remain biotrophic, but others are biotrophic only at the initial stage of the disease. As a result Vanderplank proposes that specificity depends on a process of recognition which is mechanistically linked with biotrophic nutrition. Thus, the specific pathogen protein has two functions, one catalytic, being equivalent to an elicitor or toxin, the other an ability to copolymerize. When the specific pathogen copolymerizes with the host protein, the catalytic action of the pathogen protein is annulled, the infected cell remains alive and a biotrophic relationship results; the major source of
nutrition being derived from the deregulated synthesis of the host protein. If the protein from the pathogen is not polymerized, it remains catalytic, upsetting the normal function of the host cell and therefore preventing functional biotrophy (ie it causes infected cells to die). In the absence of appropriate nutrition, the pathogen starves and its further development is prevented; the host plant becomes resistant. Thus, early stages of symbiosis could be thought of as a continuous biotrophic relationship between the two symbionts. The fact that lectin-mediated attachment of bacteria to host cell wall induces a compatible reaction in rhizobia infection, but an incompatible reaction in bacterial diseases, suggests that specificities are determined not by attachment per se, but by the interactions that precede or follow. During this regulatory interaction, rhizobia induce accessibility in host cells by their capability to suppress the host defence reaction, which they acquired during evolution toward symbiosis. On the contrary, pathogenic bacteria fail to do so, perhaps because their genetic apparatuses are loaded to produce many stimuli unnecessary for peaceful coexistence. Molecular recognition and specificity determined at the host-pathogen interface are much more complex than have been presumed. Nevertheless, it may be stated that the signal compounds produced by pathogens, such as toxins, suppressors, inducers, and elicitors, interact with recognition molecules, such as receptors or lectins located in or on the host cell wall or cytoplasmic membrane, and eventually induce the host cells to become accessible or inaccessible to pathogens depending on the intrinsic affinities and quantitative balance among interactive molecules. In cells induced to become accessible, pathogens as well as some non-pathogens (eg Rhizobium) are able to coexist in harmony.
In the induction of inaccessibility, on the other hand, cells are conditioned to reach an excited state, leading to an activation of genes for resistance and construction of physical and chemical barriers as a local response. This sort of reaction is similar to the events described for strain ANU2861, and it is possible that the single mutation in strain ANU2861 has induced inaccessibility at the plasma membrane by an inability to copolymerize with host protein (see also Vanderplank 1982). Certainly the events in strain ANU2861 infection of siratro parallel resistance responses. For example the induction of osmiophilic droplets, which may represent localized accumulation of phytoalexins or polyphenols (Gnanamanickam and Patil 1977). Polyphenols have been demonstrated to be potent inhibitors of enzymes which may limit the spread of bacteria (see Anderson 1982).

Understanding specificity is important for both practical and conceptual reasons. First and foremost, a complete analysis of the problem will allow us to devise more precise and reliable ways of controlling plant disease. In addition, insights into the nature of host-pathogen specificity are likely to prove relevant to compatibility phenomena in other biological systems, such as pollen-stigma and Rhizobium-legume interactions.

It should be appreciated that host-pathogen specificity involves several separate and probably different phenomena. While the specific mechanisms controlling the outcome of the interaction of different microbe-plant confrontations may differ, there may well be a common framework underlying them.
13.2.3 RELATION OF PERMEABILITY ALTERATIONS TO PATHOLOGICAL CHANGES IN METABOLISM: Compatible and incompatible combinations.

The concept that the nature of permeability changes induced by a pathogen determines whether a compatible (susceptible) or an incompatible (resistant) reaction will occur in a given plant-pathogen combination was developed by Thatcher (1939, 1942, 1943). This concept was based on the rationale that increases in permeability observed in compatible combinations would favour the growth and ramification of the pathogen by making water and nutrients freely available, whereas decreases in incompatible ones would restrict supplies of such substances and hence limit pathogen development. Thatcher speculated that some delicately balanced physiological factor, operative over a narrow critical range, determined the nature of disease reactions. He postulated that two pathogen-produced enzymes - one a protease, the other a lipase - might by their action on membrane proteins and lipids produce opposite effects on host cell permeability. If one or the other of these enzymes were inactivated by some substance at the protoplast surface, permeability would be increased or decreased depending upon which enzyme remained active. Wheeler & Hanchey (1968) suggested that the ability to resist and to recover from the effects of permeability-disrupting pathogenic agents might be the crucial factors which determine disease reactions. It is quite conceivable, therefore, for similar events to occur during the infection of legumes by Rhizobium, and that such factors are crucial in determining the induction of the symbiosis.

These ideas are especially interesting in respect to strain ANU2861. If, as suggested in Chapter 11, the strain has a disfunction in an
ability to alter the host cell membrane potential in a way conducive to setting up a symbiosis, then in normal infection there may be highly specific reactions that occur at the plasma membrane as host cell wall degradation ensues. It will be interesting to know how many Nod− interactions in the general Rhizobium-legume system are in fact incompatibilities at the plasma membrane (and thus Hac+ Its− Nod− interactions).

13.2.4 ELICITORS OF PHYTOALEXINS

Phytoalexins are antimicrobial compounds of low molecular weight that are both synthesized by and accumulated in plants after exposure to microorganisms. The ability to degrade and tolerate a phytoalexin may be required for virulence of a plant pathogen. Phytoalexin accumulation can be an effective mechanism of disease resistance in plants (Hahn et al. 1980).

There is evidence to suggest that pathogens may be able to secrete molecules to specifically inhibit the synthesis of phytoalexins in their hosts (Darvill & Albersheim 1984). Such specific inhibition might be accomplished by any of several mechanisms, such as by secretion of an effector molecule that activates a repressor of a gene encoding an enzyme required to synthesize the phytoalexins, or by secretion of molecules that inhibit a required enzyme. There is evidence to suggest that cell membranes are the site of action of phytoalexins (see Darvill and Albersheim 1984).
13.2.4.1 ELICITORS OF PHYTOALEXIN ACCUMULATION

Analyses of the mixture of elicitor-active glucan fragments from fungal cell walls (Phytophthora megasperma) indicated that they consisted primarily of 3-, 6-, and 3,6-linked glucosyl residues linked in the beta configuration (Albersheim and Valent 1978). Branching in the glucan carbohydrate was shown to be necessary for elicitor activity by the observation that largely unbranched B-3-linked glucans (eg laminarin) possessed very little or no elicitor activity. Other work (Ossowski et al. 1983) on the determination of the exact molecular structure of the smallest oligoglucoside possessing elicitor activity isolable from the P. megasperma B-glucan has shown that a very specific structure is necessary for a hepta-B-glucoside-alditol to be active as an elicitor of phytoalexins. Only a very small structural change can result in loss of all biological activity. These glucan elicitors isolated from P. megasperma are not race specific (Albersheim & Valent 1978); they are effective phytoalexin elicitors in tissues of other plant species. In addition, glucan elicitors with identical properties have been isolated from other sources, eg Saccharomyces cerevisiae. Thus, soybean plants, for example, are capable of responding to a wide range of fungi containing B-glucans in their cell walls, and the structures of the active glucans are not race- or even species-specific.

It has been reported that soybean cell walls contain enzymes, eg B-glucosyl hydrolase, that can degrade B-glucan elicitors (Cline and Albersheim 1981a,b). It has been suggested that such enzymic degradation would minimize the physiological importance of the B-glucan elicitors. This criticism is not valid because the hepta-B-glucosides are by far the most potent elicitors yet described.
Oligogalacturonide elicitors of cell wall origin are widespread in the plant kingdom (Cline & Albersheim 1981b).

An interesting synergistic effect has been observed between the B-glucan elicitor of _P. megasperma_ and the endogenous elicitor of soybean tissues (Cline & Albersheim 1981b). Concentrations of each of these elicitors, which by themselves are too low to elicit significant levels of phytoalexins are effective elicitors when acting in concert. Because elicitors have a profound effect on gene expression in receptive cells, it would appear to be advantageous for plants to require two simultaneous signals for maximum sensitivity in order to discriminate between tissue damage resulting from physical vectors, such as wind, which would activate only endogenous elicitors, and damage by fungi, which would activate endogenous elicitors and provide a second signal, the hepta-B-glucoside elicitor. Bacterial pathogens and fungi lacking hepta-B-glucoside elicitors could enhance phytoalexin accumulation by augmenting the amount of endogenous elicitor released from the walls of damaged cells by secreting oligogalacturonide-releasing enzymes.

Glucans isolated from compatible races of _P. infestans_ were more active in suppressing hypersensitive cell death and accumulation of phytoalexins than were those from incompatible races. These glucans are thought to have an active role in suppressing the hypersensitive reaction of host cells during the process of infection. They may inhibit the recognition of fungal hyphal wall components, and may act as determinants of cultivar race specificity in potato late blight. The reaction site for the glucans as well as the hyphal wall components may be on the protoplast surface (Cline & Albersheim 1981b).
In conclusion, plants seem to have inherent defence systems against infectious agents. Pathogenic agents, on the other hand, probably either have an acquired ability to invade particular host plant tissues without eliciting effective resistant responses or have the ability to tolerate resistant reactions of host tissues. Although there is not yet enough evidence to draw a general conclusion, each combination of host and parasite seems to have individual tactics to elicit or suppress the defence reaction of plant tissues at various steps in the sequential process.

These ideas fit in well with results in this thesis especially when comparing strain ANU240 with strain ANU2861, and especially in view of the knowledge that both Rhizobium and Agrobacterium species synthesize β-1,2-linked glucans (see Bauer 1981). Such glucans have not been reported in any other group of bacteria. Bauer (1981) suggests that it is biologically most intriguing that both Rhizobium and Agrobacterium, but not other bacteria, share the ability to synthesize these unusual, low molecular weight (ca 3000 daltons) and probably cyclic polysaccharides. As yet, no function has been indicated for these glucans, although Zevenhuizen and Scholten-Koerselman (1979) suggested that 2-linked glucan was firmly attached to the bacterial cell wall and may serve to mask outer membrane determinants. Other workers (Amemura & Higashi 1982) have demonstrated the presence of a leachable 2-linked glucan located in the bacterial periplasm, not the outer membrane. Another type of glucan has been discovered recently in culture filtrates of R. japonicum (Dudman and Jones 1980). Two closely-related glucans with terminal 3-linked, 6-linked, and 3,6-linked glucosyl residues were identified, similar to those described
for *P. megasperma* above, but quite distinct from the 2-linked glucans. It is interesting that soybean is host to both *P. megasperma* and *R. japonicum*. One of these Rhizobium glucans (either the 3- or 6-linked) acts as an elicitor of phytoalexin synthesis (ibid.). The observation that a Rhizobium polysaccharide elicits an inducible defence response in the host clearly raises the question of how root hairs are able to maintain intimate contact with their hosts in the face of both passive and inducible host defenses. In light of the results of strain ANU2861, which has a normal exopolysaccharide structure but produces significantly larger amounts of unpolymerized polysaccharide repeat units (oligosaccharides) (John Redmond pers. comm.) it is tempting to hypothesize that:

1. this strain produces a glucan configuration not normally produced by strain ANU240, and that this glucan not only stimulates a host resistant reaction, but also masks outer membrane determinants important in the symbiosis; or
2. produces a glucan of altered configuration that stimulates host biological activity, much as can occur in *P. megasperma* and hepta-β-glucoside-alditol described above.

Whatever the actual situation is, it is clear that by altering a single gene in the strain ANU240, a complete disruption of symbiosis can occur, suggesting mechanisms similar to the switching points hypothesis of Heath (1974) (Fig. 13.1).

### 13.3 FUTURE RESEARCH

A number of research guidelines can be formulated from the research presented in this thesis.
13.3.1 THE USE OF RHIZOBIUM MUTANTS AND TRANSCONJUGANTS AS PROBES OF THE INFECTION PROCESS

The technique of localising various infection and nodulation genes by transposon mutagenesis, and consequently cloning and transferring wild-type DNA fragments located by this transposon into Sym plasmid-cured derivatives is a promising method for distinguishing the various phenotypes associated with these genes, and consequently their possible functions in the infection process. By such techniques it is hoped that all the important genes, at least on the Sym plasmid, will be located and characterised. Such work is essential if these genes are to be manipulated in the pursuit of improved nitrogen fixation. Moreover, the implications for such work are far-reaching, as similar techniques may benefit work in fields such as beneficial fungal-plant root associations, and plant pathology.

13.3.2 THE USE OF LECTINS

It is clear from the results of Chapter 8 that there is a great deal of information about early Rhizobium-host interaction, such as recognition, that lectin probes can reveal. It will be most interesting to see what kinds of sugars can be detected on the surfaces of other legume roots and whether these correlate in any way with (for example) host range. In addition, further work with Rhizobium may reveal surface sugar characteristics involved in specificity.

13.3.3 MICROSCOPY STUDIES

The techniques used in examining the events of infection in siratro presented in this thesis hold great promise for future work in the
field. One of the most promising recent electron microscopic techniques that should be used is that of low temperature embedding and the application of lectin-gold complexes (on thin sections) for localising glycoconjugates at the infection site, and immuno-gold complexes for localising almost any kind of molecule for which antibodies can be made (see Roth 1983). Such molecules may be, for example, cyclic compounds such as glucans, or enzymes. This kind of microscopy has already been applied successfully in the Rhizobium-legume context where immuno-gold complexes were used to localise leghaemoglobin in the cytoplasm of the infected cells of pea nodules (Robertson et al. 1984b). Such techniques should be applied to a spectrum of Rhizobium-legume systems and infection stages to further establish the basis of infection. For example, to establish the exact origin of the vesicle-like bodies that accumulate at the penetration site and if these are similar or the same as the vesicles that contribute to infection thread synthesis. The use of mutant and transconjugant probes with such techniques will be of considerable value.
### Module Function

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
<th>Time After Infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Attachment to root surface</td>
<td>Within minutes</td>
</tr>
<tr>
<td>2</td>
<td>Multiplication on the root surface (rhizoplane)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Random erosion of the root surface</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Recognition mediated by sugars/glycoproteins</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Root hair tip attachment</td>
<td>Within 2 hours</td>
</tr>
<tr>
<td>6</td>
<td>Activation of hair curling genes - localized prevention of hair wall microfibril polymerization causing asymmetric hair growth</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Branching of root hairs</td>
<td>2 - 12 hours</td>
</tr>
<tr>
<td>8</td>
<td>Distortion of root hairs</td>
<td>12 - 20 hours</td>
</tr>
<tr>
<td>9</td>
<td>Colonization of the pocket beneath the curl</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Gradual browning and thickening of outer hair wall</td>
<td>12 - 20 hours</td>
</tr>
<tr>
<td>11</td>
<td>Degradation of the hair wall matrix and loosening of the hair wall microfibrils</td>
<td>20 - 22 hours</td>
</tr>
<tr>
<td>12</td>
<td>Penetration of the zone between hair wall and plasma membrane by bacterial colony growth pressure</td>
<td>22 - 24 hours</td>
</tr>
<tr>
<td>13</td>
<td>Communication with the host of the plasma membrane</td>
<td>22 - 24 hours</td>
</tr>
<tr>
<td>14</td>
<td>Nodule initiation - cell division commences below the infection site</td>
<td>22 - 24 hours</td>
</tr>
<tr>
<td>15</td>
<td>Infection thread synthesis by redirection of hair tip growth capabilities</td>
<td>24 - 40 hours</td>
</tr>
<tr>
<td>16</td>
<td>Orientation of infection threads towards basal part of the hair cell</td>
<td>40 - 42 hours</td>
</tr>
<tr>
<td>17</td>
<td>Infection thread penetration of the dividing cortical tissue</td>
<td>42 - 52 hours</td>
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<tr>
<td>18</td>
<td>At the moment of bacterial release, communication with the host to coordinate peribacteroid membrane synthesis with release of bacteria from thread</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>Division of rhizobia within peribacteroid membrane</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>Development of rhizobia into bacteroids</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>Separation of bacteroids into single packages (temperate legumes)</td>
<td></td>
</tr>
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### Infection and Nodule Formation

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REFERENCES


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