APPENDICES

1. Synopsis

2. List of publications
The scarcity of resources for the manufacture of nitrogen fertiliser and other associated problems have stimulated a reconsideration of current agricultural practices. As a consequence, there is renewed interest in the exploitation of biological nitrogen fixation as a major source of nitrogen input in agriculture. Biological nitrogen fixation is the exclusive domain of certain procaryotic organisms which fix $N_2$ either in the free-living state or in association with eucaryotes. The most important nitrogen fixing systems are the nodulated legumes which approximately account for almost half of the annual quantity of nitrogen fixed by biological systems. Leguminous plants establish an intimate symbiosis with nitrogen-fixing soil bacteria of the genus *Rhizobium*.

In recent years, many aspects of the biochemistry, physiology and genetics of legume-*Rhizobium* symbiosis have begun to be elucidated. Synthesis and expression of the enzyme nitrogenase *ex planta* by *Rhizobium*, widespread location of *nif* and *nod* genes on plasmids and identification of certain plant gene
products specific to symbiotic state are some of the major findings. In spite of these impressive gains, knowledge of the development of diazotroph-plant associations remains inadequate. Host specificity and mechanisms of recognition have been major areas of concern for many years. Events such as root hair curling, host cell penetration and infection thread formation probably represent different levels of recognition comprising either overlapping or distinct molecular mechanisms. Recent investigations on several legumes indicate that host responses leading to infection and nodulation are initiated in less than 2 to 4 h after inoculation of rhizobia. Such interactive root epidermal cells remain responsive towards Rhizobium for only a short (3 to 4 h) period. At present, our knowledge of legume-Rhizobium interactions is limited to post-infection events. It is important to know what happens within the above specified time period to resolve the recognition riddle. This thesis mainly examines such early legume-Rhizobium interactions.

Using the facile pouch inoculation procedure, studies have been carried out in the legumes Vigna sinensis, Vigna radiata, Arachis hypogaea and Medicago sativa to locate specific sites of infection, to establish the transient nature of these sites and to discern the overall nodulation pattern. The transient nature of the infectible root epidermal cells of Vigna sinensis...
was analysed in greater detail. Various physiological and biochemical aspects of early interactions have been examined to arrive at some of the likely sequence of responses between the Rhizobium and legume root prior to root hair curling. The thesis contains a general introduction, three chapters describing the experimental results and the conclusions arising therefrom, followed by a summary.

First chapter describes in detail, the 'pouch inoculation' methodology. Using this procedure infectible sites were localised and further studied in the legumes, Vigna sinensis, Vigna radiata, Medicago sativa and Arachis hypogaea. A high proportion of cowpea, mungbean and alfalfa seedlings consistently nodulated in that region of the root which had no root hair (NRH) at the time of inoculation with rhizobia, i.e., the region between the root tip and the smallest emerging root hair. The root cells in the region were found to remain susceptible to infection by rhizobia for only a short period of time. The appearance and loss of susceptibility to rhizobium infection followed a distinctive pattern of development. The initially susceptible region of the root became progressively less susceptible to infections as indicated by experiments where inoculation was progressively delayed.

The above assay system which enables resolution of the nodule initiation events separated by just few hours was used extensively for comparing the effect of different parameters such
as culture age, percentage of capsulated cells and inoculum strength (cells mL⁻¹ plant⁻¹) on the speed with which nodulation is initiated. The seedlings inoculated with *Rhizobium* sp. strain 1001 in stationary growth phase initiated infections at a much slower rate than the cells from log phase. There was positive correlation between faster nodulation and the percentage of capsulated cells of *Rhizobium*. Similarly, faster and more effective nodulation occurred at higher inoculum densities, with optimum strength being 10⁸ cells mL⁻¹. Although log phase rhizobia were more efficient in initiating nodulation, they showed only as much binding efficiency as stationary phase culture. Cells of either phase did not show any preferential binding to the infectible sites on the legume root. Moreover, *Rhizobium* sp. strain 1001 showed similar binding efficiency, to roots of cowpea, mungbean, alfalfa and 'methi' (*Trigonella foenum-graecum*). The non-specific binding of *Rhizobium* sp. strain 1001 to cowpea and other legume roots apparently discounts the putative existence of specific plant root protein (lectin) mediated rhizobial attachment, supposedly regulating infection process. The results clearly draw attention to the complexity and importance of early interactions between legumes and rhizobia.

The second chapter presents data which indicate the significant role of the host in early interactions. Preinfection events were analysed by studying the different nodulation behaviour of two
rhizobial strains, *Rhizobium* sp. strain 1001 (local isolate) and *Rhizobium* sp. strain 32H1. Log phase cultures of *Rhizobium* sp. strain 1001, initiated host responses leading to infection within 2 h after inoculation, whereas stationary phase cultures of the strain and log phase cultures of *Rhizobium* sp. strain 32H1 took at least 7 h to trigger a discernible response. Preincubation of the slow to nodulate rhizobium cells in cowpea rhizosphere/rhizoplane condition or in cowpea root exudate could obviate the delay in nodulation response. Complete characterisation of the active substance(s) in root exudate awaits further work, but the experiments described here reveal that the substance(s) are probably of high molecular weight and devoid of any (haem)agglutination activity. Nevertheless low levels of various glycosidase activities could be detected in cowpea root exudate, among which α - D- mannosidase was the major one. When stationary phase rhizobia were incubated for 4 h or more in cowpea root exudate, large proportion of cells formed capsule around them. The increase in cell-surface polysaccharide content showed, up to a limit, a positive relationship with the increase in percentage of plants scored for faster nodulation response. However, pre-incubation of rhizobia in cowpea root exudate had no effect on its binding to roots.

Root exudate of cowpea plants grown in the presence of 5mM $\text{NH}_4^+$ could neither stimulate polysaccharide synthesis by slow to
nodulate rhizobial cells nor promote them to initiate infections without delay, indicating that there is a regulatory role of combined nitrogen in triggering preinfection events by the legumes. Root exudate of *Vigna radiata* also promoted polysaccharide synthesis by *Rhizobium* sp. strain 1001 and faster nodulation on cowpea plants, but the exudates of *Trigorella foenum-graecum* and *Medicago sativa* were not effective indicating the relative specificity of the early interactions.

The third chapter describes the characterisation of host induced alterations of *Rhizobium* sp. strain 1001 and 32H1. The changes in capsular polysaccharides (CPS) during incubation in the root exudate were ascertained by scanning electron microscopy, cellulose membrane electrophoresis, ion exchange chromatography and gas chromatography. The CPS of log phase cells of *Rhizobium* sp. strain 1001 were altered within 6 h of incubation in cowpea root exudate. Electrophoretic or DEAE-Sephadex separation of native CPS yielded two fractions while CPS of the cultures incubated in root exudate (host induced CPS) yielded three fractions. The relative proportion of sugars in the total CPS were different. The host-induced CPS contained two new sugars, arabinose and xylose, in addition to the native components, mannose, glucose and galactose. The above mentioned alterations in the CPS composition of log phase cells of *Rhizobium* sp. strain 1001 begin by 4 h of incubation in the root exudate whereas the stationary phase cells of the rhizobium,
as also log phase cells of less competent strain such as Rhizobium sp. strain 32H1 needed much more incubation time. The results indicate that log phase Rhizobium sp. strain 1001 is able to initiate faster nodulation apparently because such cultures respond to the root exudate expeditiously.

As in the case with whole rhizobial cells, the host induced and native CPS isolated from the rhizobial cells neither showed preferential binding to host root nor did the CPS altered the binding of rhizobia. However, root hair curling induced by host induced CPS was more pronounced than native CPS. Additionally, the cowpea root epidermal cells (in the NRT region) were able to recognise host induced CPS as signal (elicitor) compound and in response showed de novo synthesis of specific plant proteins. The response was specifically elicited by the addition of host induced CPS or inoculation of Rhizobium sp. strain 1001. The newly synthesised protein bands, visualised by SDS-polyacrylamide-gradient gel autoradiographs, were absent in treatments where plants were either uninoculated or grown with NO₃⁻ or NH₄⁺. Concurrently, there were major alterations in rhizobial protein turnover during incubation in root exudate, when de novo synthesis of proteins, of subunit molecular weight 2x10⁵ and 0.4x10⁵ was observed.

The last part of the thesis is a summary consisting of brief
discussion of the results and major conclusions derived therefrom. This is followed by the literature cited in this thesis and the list of publications.

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