Chapter 4

Discussion
Lectin containing plants have been found in many botanical groups including mono- and dicotyledons, molds and lichens, but most frequently they have been detected in *Leguminoseae* and *Euphorbiaceae*. They may exist in various tissues of the same plant and have different cellular localizations and molecular properties.

4.0 IDENTIFICATION AND PURIFICATION OF LECTIN

The lectins are extremely useful tools for investigating the carbohydrates on cell surfaces and they function as recognition molecules in cell–cell interactions in a variety of biological systems (Sharon and Lis 2004). Large number of lectins has been described from various parts of different plants (Borrebaeck 1984, Makkar *et al.* 1998, Pueppke 1979, Pueppke and Bauer 1978). If lectin molecules are present in a plant, they may be distributed in all parts of the plants equally or their presence may be restricted to selected parts only. There are reports which showed earlier no heamagglutinating activity (HA) or negligible HA in the vegetative parts of several plants examined (Cazal and Lalauire 1952, Makela 1957a and 1957b, Pueppke 1979, Loris 2002). The results of our study demonstrated the presence of HA activity in extracts prepared from various parts of *S. aculeata* including the vegetative tissues. The expression of lectin activity in *S. aculeata* appeared to be developmentally regulated as the extract of 2-3 wk old plant showed higher HA score than those obtained with extracts of 1 or 4 wk old plants (Table 1). There are several reports which showed that the legume lectins are not restricted to seeds or roots only but may be present in other vegetative tissues at different stages of plant development (Cazal and Lalauire 1952, Krupe and Ensgraber 1958, Howard *et al.* 1972, Bohlool and Schimidt 1974, Talbot and Etzler 1978, Gatehouse and Boulter 1980, Hosselet *et al.* 1983, Borrebaeck and Mattiasson 1983, Borrebaeck 1984, Van Damme and Peumans 1989). In addition, lectins have also been detected in the vegetative parts of plants belonging to other families such as the *Solanaceae* (Ghanaekar and Perombelon 1980, Kilpatrick 1980), *Gramineae* (Allen *et al.* 1973), *Euphorbiaceae* (Premaratna *et al.* 1981) and some monocots (Tokuyama 1973, Mishkind *et al.* 1983).
The HA activity of *S. aculeata* extracts was inhibited by glucose, therefore, SSA, SStA, SRA and SNA were purified by affinity chromatography using sephadex G-50 as matrix and characterized. Purified SRA, SStA, and SSA moved as single protein bands corresponding to molecular mass of 39 kDa. The native gel of root SRA showed a single protein band at 83 kDa which indicated that root lectin may be a dimer composed of two identical subunits of 39.0 kDa each. The SNA showed the MW of 50 kDa on SDS gel. The observation indicated that the lectins isolated from different parts of *S. aculeata* may differ from each other.

The comparative studies on seed and vegetative tissue lectins carried out earlier by Etzler (1994) had shown that lectins may be either identical or quite different as in peanut system. The vegetative tissue lectins SL I and SL II were isolated from peanut found to be quite different and were present in 3 wk old stem tissue of plant (Singh 1995). It was of vital importance to establish carbohydrate binding specificity of lectin purified from *S. aculeata*. Our results indicated that SSA, SRA, SStA and SNA belonged to Makela’s group III of lectins like other well known mannose/glucose specific lectins. HA activity of SSA, SRA and SStA also inhibited in the presence of galactose indicating C4 hydroxyl group of carbohydrate was not very important in lectin binding. The SSA and SRA lectin were specific to α-linked glucose/mannose as shown by inhibition of these lectins with 6-methyl-α-D mannopyranoside and failure of inhibition with cellobiose.

4.1 CHARACTERIZATION OF *S. ACULEATA* LECTIN

The *S. aculeata* showed strong HA activity. Addition of CaCl₂, MnCl₂ and MgCl₂ does not altered heamagglutination activity indicating that the lectin activity of SSA, SRA, SStA and SNA was not dependent on metal ions. In this respect, the *Sesbania* lectin were akin to peanut agglutinin (PNA) isolated from peanut seeds, however, they differed from peanut root lectin PRA II which required metal ion Mn²⁺ for its optimal activity (Kalsi et al. 1992). There are several other studies where it has been shown that many lectins require metal ions such as Ca²⁺ and Mn²⁺ for the maintenance of conformation or binding to carbohydrate and exhibiting the lectin activity (Sharon and Lis 1989). The lectin activity of SRA, SStA and SNA was found to be independent of metal ion concentration. Oliveira *et al.* (2002) reported that HA
activity of the *P. capillacea* lectin isolated from red marine alga *P. capillacea* was not dependent on divalent cations.

4.2 **RHIZOBIUM OF S. ACULEATA**

The infection of leguminous plants roots by rhizobia leads to the formation of N$_2$ fixing nodules. This has been a useful phenomenon for improving the fertility of soil. Earlier it has been shown that peanut root lectin (PRA II) played a key role in legume-*Rhizobium* interactions (Kalsi et al. 1992, 1995, Jayaraman and Das 1998). The bacterial surface components have been reported to play an important role in root infection and nodule formation (Robertson and Farnden 1980). The wide spread use of *Sesbania* as a green manure led us to think about the possible role of *Sesbania* infecting rhizobia in improving the productivity. We isolated and identified the rhizobia from *S. aculeata* nodules for the first time. The 16S rRNA gene data search showed that *S. aculeata* specific *Rhizobium* was *Sinorhizobium saheli*. The isolation of different rhizobial strains were reported by many groups (Priefe 1989, Lagares et al. 1992, Lopez-Lara et al. 1996). There have been attempts to prepare mutants of several strains of *Rhizobium* used for improving fertility of soils (Zambello and Filho 1981). Thus we carried out mutagenesis experiment with a view to obtain strain with better infectivity to *Sesbania* sp. or other leguminous plants. The Tn5 mutagenesis resulted in 1.0 mutant in 3.33 x 10$^5$ *Sinorhizobium* cells. The frequency of occurrence of spontaneous kanamycin resistant mutants of peanut specific strain NC92 was recorded very high (1 mutant in 10$^5$-10$^6$ kanamycin sensitive cells) after pGS9 mutagenesis (Wilson et al. 1987).

4.3 **EFFECT OF MUTANTS ON GROWTH AND NODULATION IN S. ACULEATA**

The results of current study revealed that the *S. aculeata* plants grown in presence of SB2M3 mutant developed significantly more number of nodules as compared to those grown with wild strain SB2. The lack of naturally occurring nitrogen in most soils has been shown to establish symbiotic association between *Rhizobium* and plants belonging to the family leguminosae. It has been demonstrated that several factors associated with *rhizobia* are important in nodulation. Horvath et al. (1987) identified that a nodD gene from the wide host-range *Rhizobium* strain MPIK3030 (termed nodD1) was essential for nodulation in *Macroptilium atropurpureum* (siratro). In most of the *Rhizobium*-plant interactions studied, other active substance have been
identified as lipooligosaccharides and lipochito oligomers; the nod factors. The
synthesis of nod factors is regulated by the nodulation genes (Lerouge et al. 1990,
Spaink 2000, Truchet et al. 1991). Other genes which have been found to be directly
involved in symbiotic nitrogen fixation are fix, nif and hsn (Johnston et al. 1978,
to be spontaneous as the soil used was deficient in N₂ supply.

Rhizobia infect their respective host plants and induce root or stem nodules
using several different mechanisms. Infection through root hairs has been a commonly
seen mechanism with most legumes (Hadri et al. 1998). Rhizobia can also invade the
host plant through wounds, cracks or lesions caused by emergence of secondary roots
(Booger and Van Rossum 1997). In these cases, rhizobia spread intercellularly. There
are instances where the same Rhizobium might infect one legume through root hairs and
another via cracks or wounds (Sen and Weaver 1988). In legumes such as Arachis
hypogaea (peanut) and Stylosanthes sp, crrosyrmions infected their hosts by “crack
entry” (Chandler 1978, 1982). Rhizobia may initiate infection of the host via cavities
surrounding adventitious root primordia on the stems of Sesbania, Aeschynomene,
Neptunia and Discolobium (Boivin et al. 1997a). The stem and root nodules of Sesbania
rostrata are induced following crack entry at the base of secondary roots (Dreyfus et
for invading S. aculeata by SB2 or SB2M3 has not been studied. However, the crack
entry or information via cavities as reported in S. saheli may be operational.

In the present study, we looked for the efficiency of mutant strains in nodulation.
The S. aculeata plants were inoculated with different mutant strain (SB2M1, SB2M2,
SB2M3, SB2M4 and SB2M5) and compared with wild strain SB2. It was observed that,
out of all the mutants tested, SB2M3 was best nodulating strain, as the number of
nodules and the mg of nitrogen fixed per g of nodules increased significantly as
compared to other mutants and the wild strain SB2.

4.4 CHARACTERIZATION OF MUTANTS

Usually less mucoid colonies obtained by Tn5 mutagenesis were reported to
have defect in LPS molecules (Priefer 1989, Lagares et al. 1992). The mutant obtained
in present study was less slimy than the wild strain (Fig 15). The results of Sudan black
B staining showed that SB2M3 took higher amounts of dye. Further, it could not grow
in the presence of deoxycholate. These results indicated that there was an alteration in its outer membrane especially the LPS of mutant SB2M3. The outer membrane of the gram-negative bacteria plays a major role as an exclusion barrier against a number of potentially harmful compounds. The higher uptake of the dye by the mutant strain may be precisely due to defect in the synthesis of EPS as suggested in case of *Bradyrhizobium* by Liu et al. (1998). The synthesis of EPS and LPS in SB2M3 was indeed reduced significantly which might have permitted the dye to get in through the wall.

The SB2M3 showed very strong autoagglutinating properties. The alteration in carbohydrate production was also correlated with autoagglutinating behavior of a number of mutants (Park and So 2000). In our study, the results of hydrophobocity experiments revealed that SB2M3 was not hydrophobic in nature as the cells dispersed mostly in aqueous phase and not in organic phase. Auto agglutination generally occurs when bacteria with hydrophobic cell surfaces tend to adhere to one another (Stanley and Rose 1967). The LPS mutant of several bacterial species have been shown to be highly hydrophobic (Barness et al. 1988, Titarenko et al. 1997) and their hydrophobicity may be associated with loss of O-antigen part of LPS as in case of *B. japonicum* (Park and So 2000). Since the SB2M3 showed autoagglutination but restricted hydrophobicity, it appears that SB2M3 remained unaltered though there was change in surface molecules. The O-antigen part of the LPS molecules was also supported by the finding that O-antigen part of SB2M3 displayed rough colony morphology on YEM plates.

Here, we investigated the possible role of the cellular envelopes of the best nodulating strain namely SB2M3 and compared with the LPS of wild strain SB2 in different environmental conditions such as alkalinity, salinity etc. of the soil. The SB2M3 was found to be altered in their surface polysaccharides including EPS and LPS. The analysis of the LPS and EPS composition by colorimetric method indicated that the mutant strain had lower yield of hexose and uronic acids than the parent strain SB2 which indicated that mutant strains were deficient in the synthesis of LPS.

The EPS and LPS content of mutant strain SB2M3 was found to be significantly less than the wild strain. These changes were also observed on SDS-PAGE of LPS and EPS of SB2M3 and SB2. When LPS and EPS of SB2M3 and SB2 were electrophoresed, more number of bands were observed in the LPS I and EPS I region of SB2 than
SB2M3 indicating higher MW LPS and EPS were lost in mutant strain SB2M3. Although we could not specify the loss of O-antigen, components of LPS I region of SB2M3 were grossly affected by Tn5 mutagenesis. The LPS II and EPS II region of SB2M3 showed less remarkable changes than LPS I and EPS I region indicating no significant loss of lower MW EPS and LPS of SB2M3.

Comparative analysis of protein profiling of SB2 and SB2M3 by 2-Dgel electrophoresis showed less number of protein spots in the case of SB2M3 than SB2 on 3/10 pH strips. Thus the result indicated that there was loss of some protein spots in SB2M3 which may be supported by our findings that the surface polysaccharides of SB2M3 has changed, since the hexose and uronic acid content of LPS and EPS of SB2M3 was found to be less than SB2.

4.5 EFFECT OF SALT STRESS ON GROWTH

The changes in the structure of LPS have been considered to be as adaptation process to different environmental condition such as pH, temperature, oxygen concentration and osmotic pressure (Bhat and Carlson 1992). However, the physiological meaning of these alterations is unclear. Zahran et al. (1994) found changes in LPS I only in salt sensitive strains or in moderately salt tolerant ones. Salinity and pH are thought to be significant environmental factors determining the persistence of microorganisms in the soil. Most of these bacteria are very sensitive to a soil water deficit, which adversely affects their nitrogen fixation capacity and hence, the productivity of the whole legume plant (Miller and Wood 1996). It has been estimated that 23% of agricultural soils are affected by problems related to high salinity. Most crops are sensitive to relatively low levels of salinity and in the case of legumes, there has been an additional problem because not only the plant but also the symbiotic bacteria are sensitive to salinity both in free living stage and also during the symbiotic process (Lloret et al. 1995). The present study determined the effects of different salt concentrations on S. saheli SB2 and its mutant SB2M3.

The growth curve of SB2M3 and SB2 showed that SB2 was fast growing strain as compared to SB2M3. The log phase of SB2 was from 20-60 h whereas SB2M3 was slow growing having log phase from 72-120 h. It was observed that with the increase in NaCl concentration, the growth of the SB2 was retarded while that of the mutant strain, SB2M3 appeared to be unaffected up to 1.5 M NaCl concentration indicating the halotolerant nature
of SB2M3. We do not know the factors that made SB2M3 halotolerant, however, the ability of bacteria to grow well in high salt concentration has been shown to be dependent on the accumulation of internal solutes which maintain the osmotic balance of the cells and thus counteract the outflow of water molecules (Miller and Wood 1996, Imhoff and Rodriguez Valera 1984). These internal solutes comprise a heterogeneous group of organic compounds including polyols, sugars, amino acids, betaines and ecotines (Galinski 1995). Many rhizobia respond to salt stress by increasing the intracellular concentration of K⁺ and glutamate (Miller and Wood 1996). Reports on halotolerant strains had indicated the incorporation of negatively charged phospholipids such as phosphatidylglycerol and cardiolipin which are important for maintaining the membrane stability (Ohno et al. 1979).

Lloret et al. (1995) reported that the rise in NaCl content in the medium resulted in decreased growth rate of Rhizobium. Salinity considerably restrains symbiotic nitrogen fixation (Elsheikh and Wood 1990a, 1990b, 1995) and, therefore, the cultivation of tolerant nitrogen-fixing leguminous crops can help in improving the fertility of the saline soils (Zahran 1997). Salt tolerance of symbiotic nitrogen fixation reported to depend both on the plant and on Rhizobium genotypes (Pessarakli and Zhou 1990, Cordovilla at el. 1995). Studies on cultivar-strain interactions indicated that the S. aculeata plants can be used as a determining factor for symbiosis tolerance (Craig et al. 1991, Soussi et al. 1999). However, the salt-tolerant Bradyrhizobium strain fixed more nitrogen than the sensitive one (Elsheikh 1990b). Marked difference was observed in nodulation and nitrogen fixation parameters between salt-tolerant and salt-sensitive strains (Kumar et al. 1999). Furthermore, a salt-sensitive Rhizobium has been correlated with ineffective nodulation in soybean (Ohwada et al. 1998). The results of current study showed better nodulation in the case of high salt tolerant strain (SB2M3) than wild strain (SB2).

One approach to understand the ability of Rhizobium to tolerate salt stress is to identify stress-induced changes in LPS (Lloret et al. 1995). As important component of the external cell wall of gram-negative bacteria, LPS may play a major role in the adaptation of bacteria to the environment (Tao et al. 1992). Also, the changes in LPS structure may affect the capacity of Rhizobium to infect the roots and form effective nodules. Till date, very little information is available on rhizobial LPS of halotolerant bacteria (Soussi et al.1999, Bhattacharya et al. 2004). The Mesorhizobium ciceri strain Rcd301 tolerated up to 1M NaCl and higher salt dosages limited its growth whereas its mutant Rcd301 HT could tolerate a salt concentration of upto 2M NaCl (Pathak 2005).
The SB2M3 could be considered to be moderately salt tolerant than its parent strain SB2. The significant differences in the LPS content was also observed in case of Sinorhizobium meliloti strain Rmd201 and its variant Rmd201a (Bhattacharya 2002). In contrast, no alteration in LPS electrophoretic profile was detected in a salt-sensitive strain of Rhizobium meliloti by Lloret et al. (1995). Therefore, no direct relationship has yet been established between salt tolerance and alteration of the LPS.

In brief, the results presented here, confirmed that mutant SB2M3 was a genetically altered salt tolerant strain of SB2. The alterations in the LPS and EPS profile suggested that these changes may constitute adaptive responses to salt, allowing normal growth for effective nodulation.

4.6 EFFECT OF pH ON GROWTH

Soil acidity has been found to be a significant problem in agricultural production in many areas of the world and limits legume productivity (Clarke et al. 1993, Bordeleau and Prévost 1994, Correa and Barneix 1997, Graham 1992). Most leguminous plants require a neutral or slightly acidic soil for growth, especially when they depend on symbiotic N\textsubscript{2} fixation (Brockwell et al. 1991, Bordeleau and Prevost 1994). It has been estimated that 40% of agricultural soils are acidic and most legumes are sensitive to low pH which affects not only the plant but also the symbiotic bacteria (Graham 1992). In plants, it is well documented that acid stress results in a rapid increase in cellular putrescine (Fujihara and Yoneyama 1993).

Cellular envelopes are the first barriers that protect the bacteria from different environmental stresses including pH. Little has been known about the biochemical and physiological basis of alkali tolerance or acid tolerance by rhizobia. We observed that SB2M3 grew well in alkaline pH, the optimum pH being 8-10. Therefore, it may be useful for enhancing nodulation in leguminous plants grown in alkaline soil. The changes in EPS, LPS and protein profiling in SB2M3 may represent the adaptive mechanisms for higher pH tolerance.

4.7 CROSS BINDING STUDIES

Nodules formation on legume has been reported to be highly specific that depends on the interaction between host plant lectins and soil bacterial polysaccharides. We observed that the lectins of S. aculeata showed efficient binding
with LPS of SB2M3. This mutant was found to be better than the wild strain not only with respect to the interaction with specific lectins, but also with the lectin isolated from other plant species. The result suggested that SB2M3 may cross infect other leguminous plants.

The SRA, SStA and SSA of *S. aculeata* had same sugar specificity. It has been established that root lectins play a crucial role in binding to only specific rhizobia and that paves the way for rhizobial entry and ultimately proceed towards nodulation (Bhattacharya *et al.* 2004, Van Rhijn *et al.* 1995). The *S. aculeata* root lectin SRA displayed maximum binding with the LPS of *Sesbania* specific SB2M3 strain. Also, it was found that SB2M3 strain was the best nodule producing strain in *S. aculeata* species.

It has been also reported that the *Sesbania* was cross infective species. Hence, the cross binding studies of *S. aculeata* lectin with the LPS of various rhizobial strain was also carried out in the similar fashion. It was observed that LPS of wild strain of cicer *Rcd301* and its mutant strain *LM* showed the maximum binding with SRA. The SSA and SStA also showed binding with the cicer specific LPS but the binding capacity was less compared to SRA. This procedure ascertained that the binding of the lectins to the LPS is a species specific manner. These results clearly indicate that legume lectins are indeed valuable tools for differentiating between host specific and non-specific rhizobial strains.