CONCLUSIONS
CHAPTER - 8

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During the course of investigation, several nodule isolates were finally identified as *Rhizobium* and assigned to a species on the basis of biochemical tests and ability to nodulate an appropriate host plant.

The *Rhizobium* isolates were subjected to screening for bacteriocin production. Out of the several rhizobial strains tested, *Rhizobium meliloti* - AT 7 was found to produce bacteriocin. Unlike bacteriocins which inhibit the same or the related organisms, the bacteriocin in
the present investigation showed broad spectrum activity which differentiates it from others reported for rhizobia.

The possibility of acid mediated inhibition was eliminated by growing the producer culture on L-arabinose medium containing 0.5% CaCO$_3$. There was no reduction in inhibitory activity. Chances of hydrogen peroxide being the antagonist was eliminated by the non-performance of peroxidase in inhibition tests. The producer culture showed immunity towards its own bacteriocin - a very important criterion for specific bacteriocin production.

The medium used for bacteriocin production proved to be an important variable. In general the growth of the producer culture was quite comparable in all the six media but bacteriocin production was best on L-arabinose and D-xylose agar medium. Mannitol Nitrate Agar, Yeast Extract Mannitol Agar, Rhizobium medium 1 and Rhizobium medium 2 were inferior to these two media. Ribose, sucrose, glucose, fructose and mannitol supported good growth of the producer culture when substituted either for L-arabinose or D-xylose on equimolar basis but on the other hand there was substantial
reduction in the bacteriocin production. On the basis of their inhibitory action on bacteriocin synthesis, the sugar substitutes can be arranged in the following sequence: Fructose > glucose > sucrose > mannitol > ribose. Combinations of these sugars with succinate and citrate resulted into further enhancement in growth but the bacteriocin synthesis remained unaffected.

The consistency of the habitat proved to be an important variable; production was not detected in L-arabinose or D-xylose broth regardless of the time of harvest, growth stage or aeration.

The bacteriocin was elicited at different temperatures (25 to 37°C) without any remarkable reduction in the yield.

The killing activity of the bacteriocin proved resistant to heat (85°C for 45 mts) and trypsin. However, after heat treatment the bacteriocin was digested by trypsin. This suggests that the bacteriocin in its native form is resistant to trypsin but heat treatment results in some change in the molecular confirmation of the protein which ultimately makes it susceptible to trypsin.
The bacteriocin was resistant to DNase, RNase and lysozyme as evidenced by their non-performance in the inhibition tests when studied separately. The bacteriocin produced by *R. leguminosarum* has been reported to be chloroform sensitive (Brussel *et al.*, 1985). In contrast to this, the bacteriocin of *R. meliloti* - AT 7 was insensitive to chloroform. The bacteriocin could diffuse through cellophane membrane (6000 to 8000 molecular weight cut off). This suggests that the bacteriocin does not belong to the conventional class of high molecular weight bacteriocins.

The spontaneous production (which minimizes variation involving inducing and non-inducing environment), broad spectrum activity (which maximizes the chances of controlling wide range of strains) and low soil binding properties (which minimizes the chances of reduced activity due to soil binding) makes it a potential antibacterial agent.

From the standpoint of competition between strains of rhizobia, the known bactericidal function of the bacteriocin could be significant in providing the bacteriocinogenic strain a competitive advantage over
other rhizobia. The results showed that *R. meliloti* - AT 7 dominated over bacteriocin sensitive *R. meliloti* strains (strain AT 5 and AT 6) during *Medicago sativa* nodulation studies in sterile soil. All singly inoculated strains grew well and also when mixed inoculated with bacteriocin non-producers. The bacteriocin resistant strain AT 10 M was not suppressed during mixed inoculation. When *R. meliloti* - AT 7 and *Rhizobium* sp. (cowpea) - AT 4 were mixed inoculated during *Vigna unguiculata* nodulation studies in sterile soil, it was observed that the presence of bacteriocin producer significantly retarded nodulation in cowpea plants. Further when bacteriocin resistant strain AT 10 M was competed with native rhizobia in non sterile soil, it was observed that the native inefficient stains outcompeted strain AT 10 M. However, when strain AT 7 was mixed inoculated with strain AT 10 M the competitive ability of strain AT 10 M was enhanced as a result of the antagonism of the native rhizobia and subsequent decrease in the number of bacteriocin sensitive competitors. Similar results were observed in plant tube experiments.
The moisture content of the soil proved to be an important variable, with the growth suppression of the bacteriocin sensitive strains AT 4, AT 5 and AT 6 being much more marked in "wet" than "damp" soil. This could be due to the availability of free liquid in the wet soil which facilitates bacterial mobility and diffusion of the inhibitory substance from producing cells to sensitive cells.

In summary, suppression of the bacteriocin sensitive strains and non-suppression of the resistant strain, provide the best evidence for the presence of functional bacteriocin in sterile and non-sterile soil.

Before embarking on the studies on the correlation between EPS production and nodulating ability of cowpea rhizobia, it was necessary to find a suitable defined medium for the strains under investigation that would allow good growth of cells and large yields of EPS. A defined medium containing 1% xylose, 0.8% sodium citrate, 0.25% sodium glutamate, 0.019% MgSO₄·7H₂O, 0.005% NaCl, 0.03% K₂HPO₄, 0.005% CaCl₂·2H₂O, 0.0006% FeCl₃, 0.03% KH₂PO₄, 0.00002% biotin, 0.00001% thiamine HCl; 0.00001% calcium pantothenate was suitable for optimum
growth and polysaccharide synthesis. All the cowpea rhizobia strains showed enhanced growth and higher EPS yield in GPM1 as compared to that in YEMA. Growth of the culture in this medium did not cause the culture to lose their symbiotic properties.

Nodulation pattern of Vigna unguiculata was markedly influenced by EPS preparations from highly infective and poorly infective strains of cowpea rhizobia. The addition of crude EPS preparations from highly infective Rhizobium sp. (cowpea) strains AT 1, AT 3 and AT 4 increased the infectivity of poorly infective mutant strains derived from the aforementioned parental strains. The extent to which the crude EPS preparations enhanced the nodulation rate and therefore, the coefficient of infectivity varied with each strain and was highest when the EPS from a highly infective strain was added.

The decrease in the coefficient of infectivity after reaching a particular value inspite of the increase in the concentration of EPS preparation from highly infective strains could be due to the high viscosities of the solutions at higher concentrations; which might inhibit the normal migration of bacteria.
On the other hand the results obtained using crude EPS preparation from *R. meliloti* AT 5 showed that the effect was not caused by a non-specific substance. Addition of *R. meliloti* - AT 5 crude EPS at the same concentration as that of EPS from highly infective cowpea rhizobia did not show any stimulatory effect on the nodulation pattern of *Vigna unguiculata*.

Further, the stimulatory effect of crude EPS preparations on nodulation rate was not affected even if dialysed and/or cetyltrimethyl ammonium bromide precipitated EPS preparations were used. The results confirm that the positive influence of the crude EPS preparations was not due to the presence of hormones since such substance would be removed on dialysis or when treated with cetyltrimethyl ammonium bromide.

Addition of crude EPS preparation extracted with ethyl acetate resulted into lowering of the CI values. This was found to be due to partial hydrolysis of EPS due to acidic pH required during extraction. This was supported by the CI values obtained after addition of acid hydrolysed EPS.
Addition of crude EPS of highly infective cowpea rhizobia grown on cowpea root exudate remarkably increased the coefficient of infectivity as compared to the EPS preparations of the strains grown on GPM1. Similarly the EPS preparations of poorly infective mutants grown on cowpea root exudate showed increased potential. In contrast to these favourable effects, crude EPS of R. meliloti strain AT 5 grown on cowpea root exudate, did not exert any positive influence on Vigna unguiculata nodulation rates indicating that the receptors on the microsymbiont show specificity.

In summary, it is quite reasonable to expect that the EPS plays some role in the 'recognition' process in Rhizobium sp. - Vigna unguiculata symbiotic association. Further, the effective infection is not only preceded by synthesis of EPS in quantity but also it should be of the right structure. Reorientation of EPS may be stimulated by the specific plant root exudate.