CHAPTER V
SUMMARY AND CONCLUSIONS

Andaman & Nicobar Islands are called biological paradise due to its rich resource of plant diversity. This plant diversity was well explored for its application in agriculture and other fields. However, the rich potential of microbial resources associated with this plant diversity was untouched till date. One such beneficial plant-microbe combination is *Rhizobium*-wild legume association. The wild legumes are very common near the shore throughout Andaman & Nicobar Islands and many of them established copiously even under the adverse conditions *viz.* marshy and swampy areas of mangrove creeks, back water lands and surprisingly some of them were seen even under sea water inundation during high tide. Profuse growth of these wild legumes under adverse condition is not possible without the stress tolerant root nodulating bacteria, i.e., *Rhizobium* spp. To tap the potential of these microbial resources associated with wild legumes a survey was undertaken in Andaman & Nicobar Islands and systematic experiments were carried out to screen the nitrogen fixing, root nodulating and plant growth promoting bacteria associated with wild legumes that are naturally growing in the extreme habitats of coastal areas. The extreme areas emphasized here is saline, saline alkaline, sodic and acidic soils in which the normal crop cannot be cultivated. The wild legumes that are naturally and abundantly growing in the above mentioned soil conditions were collected, associated putative *Rhizobium* spp were isolated, characterized for salt and pH tolerant properties. The efficient nitrogen fixing bacteria were tested for their cross nodulation with cultivated legumes *viz.* green gram, black gram and cowpea in order to tap their potential for their future application in improving the productivity of pulse crops. The summary of the findings are given below:
1. A survey was conducted in seven Islands viz., South Andaman, Middle Andaman and North Andaman, Jollybouy, Havelock, Rutland and Niel Island to collect wild legumes plants naturally growing in the sea shore, backwater lands, mangrove creeks, uncultivable lands and Tsunami affected coastal regions. A total of 36 wild legumes were collected with root intact and preserved in herbarium.

2. The soil in which wild legumes were growing was collected, brought to the laboratory and the properties of the soil viz., pH, Electrical Conductivity and Cation Exchange Capacity were analysed. The place in which sea water intrudes during high tide the soil samples were collected after receding of tide. The pH of samples tested ranged from 5.3 to 6.9, EC ranged from 0.01 to 4.5 dSm\(^{-1}\) and CEC ranged from 9.3 to 15 c.mol (+)/Kg.

3. The wild legumes preserved were recorded for plant characters and identified up to genus and species level with the help of Botanical Survey of India, Andaman & Nicobar circle. The fresh plants immediately after collection were recorded for their nodulation properties and the root nodules were separated for isolation of root nodulating, nitrogen fixing bacteria.

4. A total of 32 bacteria were isolated on Yeast Extract Mannitol Agar from the root nodules associated with the wild legumes. The growth of bacterial colonies on YEMA were circular, elevated, semi translucent, mucilaginous with entire margin. The bacteria were rod shaped, non spore formers and capsulated.

5. The biochemical properties of bacteria associated with wild legumes were studied in detail. All the bacteria were indole negative, catalase positive and non fluorescent. The bacteria showed mixed results of positive and negative reaction to MR-VP, ammonia production and TSI test.
6. The bacteria showed versatility in utilizing different carbon sources as energy sources. Seven cultures (WL11, WL12, WL32, WL33, WL34, WL35, and WL36) utilized all five carbon sources, WL26 and WL28 were the only two cultures that showed negative reaction for all carbon sources. Among the bacteria only 23, 15, 20, 21 number of isolates utilized lactose, glucose, sucrose and starch, respectively.

7. In an another test of biochemical characterization (with kit) four isolates WL1, WL8, WL13 and WL25 given positive reaction to phenylalanine deaminase test, 19 isolates out of 32 utilized citrate, 20 isolates showed positive reaction for ornithine decarboxylase test while 16 isolates showed positive reaction for urease test and only 9 cultures produced hydrogen sulphide (WL6, WL12, WL16, WL19, WL22, WL24, WL25, WL26 and WL27). Glucose and arabinose was readily utilized as carbon source by many of the bacteria, while only six isolates utilized adonitol as sole carbon source.

8. In the in vitro nitrate reduction test except five isolate viz., WL6, WL16, WL23, WL30 and WL36 all other rhizobia reduced nitrate.

9. In antibiotic sensitive assay most of the isolates showed high resistance to rifampcin while many isolates were sensitive to erythromycin and 14 isolates exhibited marked resistance to all ten antibiotics tested.

10. The analysis of cultural and biochemical properties exhibited by the isolated root nodulating bacteria showed that they were in mostly in accordance with the cultural description of *Rhizobium* sp given in Bergey’s manual.

11. In vitro test of salt tolerance showed that among the 32 root nodulating bacteria 24 grew at the salt concentration of 1000mM NaCl, but 8 rhizobial isolates failed to produce colony at 1000mM NaCl. Among the 24 bacteria 13 isolates managed
to grow at the concentration of 1400mM NaCl in the media however, only six *Rhizobium* sp (WL1, WL8, WL12, WL13, WL19 and WL20) formed colonies at a salt concentration of 1600mM NaCl. The salt tolerant property of these six isolates was highest among the reported salt tolerant property so far.

12. All 32 rhizobial bacteria showed good survival in pH range 5-9 whereas only 15 root nodulating bacteria could survive both in high acidic pH of 4 and alkaline pH of 11.

13. Out of 32 bacteria only nine cultures produced siderophore and among them WL13 and WL28 showed high amount of iron chelation. All 32 isolates were capable of producing IAA *in vitro*, with WL 1 producing higher level of IAA than other bacteria. Only six isolates *viz.*, WL1, WL8, WL12, WL18, WL28 and WL31 could solubilize phosphate on Pikovaskaya’s agar and few bacteria like WL1 possess all three PGP tested.

14. In molecular characterization of isolated bacteria, all 32 bacteria exhibited *nifH* gene by producing a band at 781 bp when subjected to PCR analysis with *nif* H1 and *nif* H2 primer pair.

15. One single band of slightly less than 1500 bp was amplified using universal bacterial 16s rDNA primer FD1 and RD1. Restriction of 16S rDNA PCR products of all the bacteria with restriction enzyme *Hinf* I and *Hae* III and the dendrogram analysis showed existence of much diversity among the *Rhizobium* associated with wild legumes of Andaman & Nicobar Islands. Both the dendrogram showed grouping of these bacteria by two major clusters and 5 sub clusters. The bacteria with highest salinity tolerance (WL1, WL12, WL8, WL13, WL20, WL28, WL31) fall into same sub cluster giving evidence for similar genotype. The only one bacteria, i.e., WL36 exhibits marked closeness with the reference culture MTCC
indicating that it may be closely related to *R. leguminosarum*, which is common among cultivated legume.

16. *Rhizobium* sp that conferred higher tolerance to NaCl and pH along with plant growth promoting ability were selected for cross inoculation experiments with *V. radiata*, *V. mungo* and *V. unguiculata* in Jensen’s medium. No cross infection and nodule formation was observed in legume seeds inoculated with WL18 and WL34, whereas the remaining seven bacteria nodulated one or more legumes. Bacterial isolate WL1 isolated from wild legume *V. marina* cross infected with all 3 legume seeds and produced microscopic nodules. These seven cross nodulated bacteria individually and in combination (consortium) taken for cross inoculation experiment with the same 3 cultivated legume crops in pot culture.

17. In green gram cross nodulation study the consortium of *Rhizobium* formed maximum number of nodules (54) followed by WL1 (49) and WL12 (37). The similar trend was noticed with the viable nodules, wet weight and dry weight of nodules.

18. In black gram, root nodulation was found more in treatment with WL1 (41), WL12 (40) and consortium (39). Number of viable nodule and nodule dry weight of WL1, WL12 and consortium were higher than that of other treatments.

19. Nodulation pattern in cross nodulated cowpea showed similar results like nodulation pattern in black gram, in addition to that the bacteria tested produced unique nodules in terms of shape, size and number. The bacteria WL1, WL12 and consortium of root nodulating bacteria produced highest number of nodules *viz.*, 59, 58, and 58, which is highest among all three legumes tested. The dry weight of nodule followed the same trend as no of nodule.
20. The nodule produced in the pot culture showed that the nodulation by tested wild
legume associated bacteria can be referred as diffused type as they are distributed
both on tap and lateral roots, with more on tap root than on lateral roots.

21. In pot culture experiment green gram cross nodulated with WL 1 exhibited higher
nitrogenase activity, i.e., 48µmol C₂H₂/h/g of nodule. Similarly green gram that
received mixed inoculums also recorded maximum nitrogenase activity i.e.,
50µmol C₂H₂/h/g of nodule followed by WL12. In black gram, plants treated with
WL1 and WL12 showed higher nitrogen fixation ability, i.e., 42.6 and 39.3µmol
C₂H₂ production/h/g of nodule, which is higher than the consortium or any other
treatment. When compared to green gram and black gram nitrogen fixation in
cowpea was observed to be slightly higher for almost all the treatments. The
maximum nitrogenase activity was recorded in WL1 treatment (56.1µmol
C₂H₂/h/g of nodule), followed by consortium application (50.7µmol C₂H₂/h/g of
nodule) and WL12 (49.1µmol C₂H₂/h/g of nodule). The analysis of data indicates
that the consortium activity was mainly contributed by WL1 and WL12 but not by
any other root nodulating bacteria.

22. Effect of cross nodulating bacteria on yield parameters were calculated by
measuring shoot and root length, plant biomass, number of pods production/plant
and number of seed production/plant. Green gram seed treated with T8 produced
plants with highest shoot as well as root length i.e., 35.4 cm and 19.4 cm,
respectively. The effect of WL12 treatment was statistically on par with WL1
treatment but both were significantly higher than other treatments.

23. The total biomass in green gram was higher for consortium treatment (5.08g)
followed by WL1 (3.95g) and WL12 (3.44g). Statistically T1 and T3 had no
significant difference on biomass production, however T1 produced higher
number of pod and seeds per plant (16 pods, 8.39 g) than T8 (16 pods, 8.23 g) and T3 (15 pods, 7.8g).

24. The shoot and root length of black gram ranged from 29.0 to 32.6 cm and 13.9 to 15.2 cm, respectively with not much significant variation among the treatments. Bacterial isolate WL1 produced the highest total biomass in black gram (6.67 g) followed by WL 12 (5.03 g) and consortium (4.72). Similarly WL 1 (20, 13.29 g) produced highest number of pod and seeds per plant followed by WL3 (18, 9.89 g) and consortium (18, 10.54 g).

25. In cowpea T1 and T8 showed marked effect on the shoot length by producing 39.2 and 40.1 cm shoot followed by treatment T3 (34.2cm), whereas the root length was significantly more in the plants treated with T8 (22.4 cm) followed by T3 (18.2 cm). Higher biomass was produced in cowpea by consortium of bacteria (12.45 g) followed by WL1 (11.41g) and WL12 (8.05g). Plants treated with mixed inoculum of bacteria also produced higher number of pods and number of seed per plant (11, 21.33g) followed by WL1 (11, 20.85g) and WL12 (9, 16.65g).

26. The total analysis of treatment effect on yield parameters showed that the plants treated with WL1 produced lengthy roots with nodules of 3-4 mm diameter and 4-8 mm length. The WL1 treated plants were also observed with thick shoot system and large canopy while plants treated with WL12 produced nodules of 1-4mm diameter and 1-6 mm of length with many secondary and tertiary roots thus forming an extensive root system with medium size of nodules that compensates efficiency of WL1. The plants treated with consortium conferred only the properties of WL1 and WL12 and thus producing higher nitrogenase activity, biomass and yield onpar with these 2 bacteria.
The 16s rDNA partial sequencing, BLAST search and phylogeny analysis of promising root nodulating bacteria i.e., WL1 and WL12 showed that they belong to the genus *Rhizobium* and their species level delineation could not be done as WL1 matched 100% with unknown species of *Rhizobium* associated with *Bursaphelenchus xylophilus* (Accession no. HM142088) and WL12 match 92% with unknown species of *Rhizobium* associated with native legumes of Thar desert (Accession no HM142088).

In the present study few novel legume-*Rhizobium* symbiosis were identified in extreme environments of Andaman & Nicobar Islands. The results showed existence of diverse population of *Rhizobium* spp in symbiosis with wild legumes. These bacteria showed extensive *in vitro* salt tolerance property, i.e., upto 1600mM NaCl and produced colonies in varied pH. The potential of these bacteria were confirmed with potculture experiments, in which 7 bacteria nodulated with cultivated legume and among them 2 *Rhizobium* spp, i.e., WL and WL12 were exceptionally good in terms of root nodulation with green gram, black gram and cowpea. These two *Rhizobium* spp showed some unique nodulation pattern with all three legumes, i.e., WL1 produced relatively bigger nodules with high N\textsubscript{2} fixing ability, whereas WL12 induced the host legume to produce more number of secondary and tertiary roots thereby increasing the surface area of root for nodulation as well as plant nutrient absorption. Eventhough the option of bacterial consortium worked on par with WL1 and WL12 it was not significantly higher in efficiency over WL1 and WL1, as the consortium mostly exhibited the potential of WL1 or WL12 but not the potential of other 5 bacteria tested.

Hence the present findings suggest that *Rhizobium* culture WL1 and WL12 can be individually applied to the cultivated legume to enhance the crop productivity. The result
shows that the effect also can be reproduced by testing of WL1 and WL12 in the problematic fields, which will have great impact in yield enhancement. In India pulse productivity is always low due to the inherent property of soils such as cultivation of crops in problematic soil. The agricultural problematic soils can be classified as saline (pH <8.5; EC >4.5; ESP >15), alkaline/sodic soil (pH >8.5; EC <4.5; ESP >15), saline alkaline soil (pH >8.5; EC >4.5; ESP >15) and acidic soil (where pH <7). The nodulation property of legume- *Rhizobium* is severely affected by these problematic soils there by limiting the expansion of area under pulses cultivation. Screening of 2 effective root nodulating bacteria in the present experiment solves this problem by efficiently nodulating and enhancing the yield of cultivated crops under these unfavourable conditions. The isolated bacteria were screened for varied acidic and alkaline pH, the EC of the habitat mimic various problematic soil and the bacteria were exposed to high salt concentration *in vitro* screening that simulate high Exchangable Sodium Percentage (ESP) of soil and in addition their efficiency were proved by statistically significant effect of cross nodulation, nitrogen fixation and yield of green gram, black gram and cowpea. In conclusion it can be stated that the present finding resulted in screening and identification of 2 novel *Rhizobium* spp that can be utilized to cross nodulate and enhance the yield of cultivated legumes *viz.*, green gram, black gram and cowpea even under adverse soil conditions, in addition these findings opened a new avenue for exploration of such unique and diverse bacteria associated with our rich plant resource to enhance the crop productivity.