CHAPTER 6
DISCUSSION

India is the biggest producer of Mung bean in the world. Nitrogen is the furthermost limiting nutrient for growth of the leguminous plants (Simon et al 2014). Many soils do not have adequate quantities of native rhizobia in relationships of number, quality and effectiveness to enhance biological nitrogen fixation (Denton et al., 2013). To confirm an optimum rhizobial population, legumes seed inoculation with an efficient rhizobial strain is essential; this helps to increase nodulation, nitrogen fixation and yield of leguminous crops (Somasegaran and Hoben 1994). It was detected that inoculation of Rhizobium spp. increases photosynthetic rate, leaf area, plant height, and dry matter production (Thakur and Panwar 1995). Inoculation of faba bean efficient rhizobial strain reduces the need of nitrogen fertilizer to attain high crop yield under low soil fertility conditions (Yousief et al., 2017). BNF has great importance as ecofriendly and cost effective way to improve soil fertility (Yousief et al., 2017). The identification of native strains able of fixing nitrogen will make available an inexpensive solution for enhancing legume production. This study was undertaken to develop a suitable Rhizobium strain for mung bean for enhanced nitrogen fixation.

6.1 Collection of root nodule, isolation and identification of Rhizobium

For collection of nodules, first healthy plants were collected from locally grown mung bean farm from Punawale, Pune. The isolated culture were characterized and identified as Rhizobium japonicum based upon nodulation test, morphological, cultural and biochemical characteristics. The culture takes six to seven days for full growth which is the characteristics of the slow growing Rhizobium japonicum (Somasegaran and Hoben 1994). Nodulation test is the confirmatory test for Rhizobium (Somasegaran and Hoben 1994). The Rhizobium
isolate was classified as fast and slow growers according to Bromothymol blue test. The similar study was carried out by (Hungria et al., 2001) and (Saeki et al., 2005) to classify *Rhizobium* as fast and slow growers. Glucose Peptone Agar test is the confirmatory test for the *Rhizobium* (Kucuk et al., 2006). In thus study isolate able to grow on GPA. It was observed that rhizobial cells do not produce gelatinase enzyme. Negative gelatinase activity is also a feature of *Rhizobium* (Hunter et al., 2007). Positive result obtained from starch hydrolysis indicates that the isolate have potential to hydrolyse starch present in the medium (De Oliveria et al., 2007).

### 6.2 Generation of Transposon induced mutants

For bacterial mating rifampicin resistant mutants were produced as previously by (Khetmalas 1996). 100µg rifampicin was added to YEMA plates for counter selection against *E.coli*. Reduction in symbiotic effect of *Rhizobium spp.*against streptomycin resistant mutants of cicer infecting chick pea plant was observed by (Dadarwal and Sindhu 2001). They found some mutants with effective nodulation to the plant and also there were significant differences among nodule forming ability of mutants from the same parent strain. Some mutants did not form nodules on root system of chick pea plant, while nodules formed by some mutants show no acetylene activity. Dadarwal and Sindhu concluded that antibiotic known to inhibit protein synthesis. The spontaneous resistance probably results in strain possesses an unknown number of multiple mutations changes some phenotypic characteristic which results in loss of symbiotic effectiveness and bacteroid differentiation. In this study nodulation and nitrogen fixation result of *Rhizobium japonicum* AVR 1 clearly indicates that there is no change in nodulating ability of rifampicin resistant mutant; so it became the candidate for conjugation experiment. In this experiment we strictly followed mating conditions because mating time and donar- recipient ratio affects
conjugation efficiency (Wahyudi et al., 1998). *E.coli* S 17 strain used in this study has the conjugal transfer genes (tra) on the chromosome and has ability of taking any plasmid that contain an origin of transfer and mobilizing it to a suitable strain (Simon et al., 1987). Suicide plasmid pKO3 was used by (O’ Connel et al.1998) to study the genetic approach of *Rhizobium tropici* to understand mechanism of foliar chlorosis (yellowing of leaves) in *Phaseolus vulgaris*. We successfully developed transposon mutagenesis system for *Bradyrhizobium japonicum* AVR 1 by using *E.coli* S17 harboring suicide plasmid pK03.

### 6.3 Screening of transposon induced mutants

This system produced variety of nodulating mutants. Transposon mutagenesis has several advantages over other methods such as chemical and physical mutagenesis.(Khan2012) Initially, transposons mark their site of insertion allowing easy isolation (Guihabert and Hoffman 2001). Secondly, mutant cells containing transposon insertion can be separated from wild using an antibiotic marker (Berg et al.1989). Secondly, for these reasons we selected transposon mutagenesis. The transposon mutagenesis of *Agrobacterium tumificiens* by biparental mating was previously done by Khetmalas 1996; the transposon frequency obtained in mating was $4.2 \times 10^{-4}$. In this study the transposition frequency was $3.7 \times 10^{-6}$. In present experiment nodulation result of transconjugate showed that some mutants with poor nodulation, some mutants with white coloured nodules and some are non nodulating. These mutants are deficient in functional interactions with plant. Study of plant-*Rhizobium* association including study of mutants lacking in functional interactions will help to increase our information about the individual organism and overall ecology of plant-microbe interactions (Neerj et al., 2009).
6.4 PCR amplification and DNA sequencing

For PCR amplification pair of primer TNP1 was used which was previously used by (Ferreira et al. 2002) to identify signs of the presence of transposon Tn1721 sub group of Transposon Tn3. The transposon Tn3 may be able to replicate and transpose (Grindley 2002) and such rearrangement might have occurred due to addition or deletion event. PCR amplification result of samples AVR040 and AVR063 detect band at 600 bp and the expected band size of primer designed by Ferriera was 611 bp. This data suggested that transposition was successfully occurred in the strains AVR04 and AVR063. The amplified PCR product was subjected to DNA sequencing by using Sanger sequencing method. Results obtained from DNA sequencing and BLAST analysis reveals that transposition was occurred in the genes Aldehyde Oxidase (AO) for AVR040 and RND Transporter for AVR063 strain. AO (EC 1.2.3.1) fits to the family of molybdenum hydroxylase. found in animals, plants and microorganisms, it catalyses oxidation of aldehydes and nitrogen containing heterocycles (Pascual et al. 2007). Aldehyde Oxidase is catalyzes in biosynthesis of two hormones abscisic acid (ABA) and the Auxin, indole3 acetic acid (IAA) via the Trp dependent indole3 pyruvic acid pathway (Koshiba et al. 1996). In general root nodule contains more auxin than non nodulated roots (Gosh and Basu 2006). The bacterial IAA synthesis has special role in symbiosis between Rhizobium and legume plant (Remans et al., 2008). Bradyrhizobium elkanii mutants lacking in IAA making formed fewer nodules on roots of soybean plant (Fukuhara et al. 1994). Indole Acetic Acid is a give and take signal in legume Rhizobium connections (Spaepan et al., 2007). Initially host produced IAA might be utilized as indication by Rhizobium to recognise the surrounding area of plant roots and modify its gene expression profile for genes concerned in initial stages of nodule
development. Bacterial IAA produced in nodules can then produces compact touch with plant cells and therefore get in the way with IAA signalling in plants. RND efflux pump facilitates bacteria/plant relations at different points that comprise the response to toxic compounds, host specificity and interspecies signalling trafficking (Carolina et al. 2013). Mutants of *Rhizobium etli* lacking the RamrAB efflux pump forms less nodules on its host plant than its wild strain (Gonzalez-Pasayo2000). Similarly the SmeAB efflux pumps play a crucial part in nodulation competitiveness in *Sinorhizobium meliloti* (Eda et al. 2011). RND type pump, BdeAB required for effective N₂ fixation in soyabean nodules caused by *Bradyrhizobium japonicum* (Lindemann et al. 2010).

Results obtained from studies performed during present investigation indicated that transposon was successfully inserted in to the *Rhizobium japonicum* AVR1 and it grows on selective antibiotic markers. PCR amplification with Tn3 specific primers confirms transposon insertion at molecular level. DNA sequencing and BLAST analysis states that Tn3 was attached on gene Aldehyde Oxidase and RND Transporter which results in transposition in the genes which leads to enhancing nodulating and nitrogen fixing ability of strain AVR040 and AVR063. The strain AVR063 shows higher nitrogenase activity with increase in N₂ fixation and nodulation, shoot fresh and dry weight without any deleterious harm to the environment.