5. DISCUSSION

Several environmental factors adversely affect the plant growth and development and final yield performance of a crop. Drought, salinity, nutrient imbalances and extremes of temperature are among the major environmental constraints to crop productivity worldwide. Development of crop plants with stress tolerance is a very important research. Recently, the scientists try to improve plant tolerance to extreme environmental conditions through the biobertilizers treatments (symbiotic nitrogen fixing bacteria, asymbiotic nitrogen fixing bacteria and mycorrhiza). *Rhizobium* population tolerance to major environmental factors than their host legumes. *Rhizobium* symbiosis with leguminous plants and fix atmospheric N₂. Symbiosis behaviour under the salt and drought conditions discussed hereunder.

5.1. Morphological characteristics of *Rhizobium*

A total thirty-one isolates were collected from the nodules of *Vigna mungo* and *Arachis hypogaea* plants in different districts of Tamil Nadu. These isolates were designated as Rhizobia on the basis of their colony characteristics, cell morphology and cultural characteristics. Differences between isolates were verified using morphological parameters. High production of mucus was identified in 35% of the isolates, 13% were white and 52% isolates were mucoid, opaque colonies. All isolates were gram negative, rod shaped and non motile. Vincent (1970) reported that the *Rhizobium* grown on YEMA medium and produced small to medium sized colonies, usually smaller than 2mm. This results were confirmed by using Bergey’s Manual of determinative bacteriology (Holt et al., 1994)
The pH of the medium during the growth of isolates was changed from 7 to 6, thus showing the production of acid which is characteristic of \textit{Rhizobium} to produce acid during growth. Similar findings were made by Baoling \textit{et al.} (2007).

5.2. Identification of \textit{Rhizobium}

In present investigation from the characterization tests, it is evident that all the isolates from \textit{Vigna mungo} and \textit{Arachis hypogaea} plants are non ketolactose producers and showed no growth in Hofer’s medium. Pure \textit{Rhizobium} isolates are unable to grow on lactose (Kucuk \textit{et al.}, 2006). \textit{Rhizobium} can not grow at pH 11 in the Hofer’s alkaline medium (Gaur and Sen, 1981).

The agrobacteria absorbed the congo red, whereas the rhizobia colonies stand out as white, mucoid and translucent. \textit{Rhizobium} showed red colour in PHB stain under microscope. Growth reactions of \textit{Rhizobium} in Litmus milk, one of the characterization test to differentiate different isolates, have been studied by various workers.

Rhizobia distinguished from Agrobacterium and confirmed that the findings of the present study have been isolated \textit{Rhizobium} species from the root nodules of \textit{Vigna mungo} and \textit{Arachis hypogaea} plants but not Agrobacterium. Similar findings were made by Kumari \textit{et al.} (2010) for the characterization of \textit{Rhizobium} isolates from \textit{Indigofora} species and confirmed that those organisms which have the above said characteristics are used to identification of \textit{Rhizobium}. 
5.3. Biochemical characterization in *Rhizobium* strains

All the rhizobial isolates were positive to the Indole, nitrate reduction, urease, catalase, oxidase and Mac-conkey agar test. All the 31 isolates were not produced H₂S and utilized citrate as a sole source of carbon.

Positive results were obtained from the starch hydrolysis assay. On subjecting inoculated plates to iodine test, clear zones around the colonies were seen and the colonies turned yellow in appearance, whereas blue color appears on no growth areas. It indicates the isolates have the potential to hydrolyze starch present occur in the medium. De Oliveira *et al.* (2007) also observed that the *Rhizobium* strains utilize the starch obtained from the different sources.

Rhizobial cells produce gelatinase enzyme and negative gelatinase activity also feature of *Rhizobium* (Hunter *et al.*, 2007). Yellow slants and red butt were obtained showing the utilizing of glucose and sucrose in the Triple Sugar Iron agar medium (Hajnaa, 1945). No such studies have been conducted on *Rhizobium* strains. Naz *et al.* (2009) also found the same findings.

5.4. RAPD-PCR Analysis

The RAPD technique was used to detect the polymorphisms among the 31 strains of Rhizobia used in this study. The number of amplified products ranged from 4-8 per isolate. In this present investigation, 31 Rhizobial isolates can be divided into 3 clusters, second cluster having 2 groups. By the RAPD analysis clear differences were observed between 31 Rhizobial isolates. The greater genetic difference among these isolates
different area from *Vigna mungo* and *Arachis hypogaea*, used for classification of Rhizobia. Ilyas *et al* (2008) characterized the *Rhizobium* strains on the basis of RAPD-DNA fingerprinting and evaluated that environmental stress may favor adaptation of strains with genetic difference. PCR-RAPD is a useful tool to conduct persistence and competitiveness studies in rhizobia strains when inoculated in soils as inoculants (Pinto *et al*., 2004). This characters can be useful for future improvement of inoculants by genetic engineering of strains with high efficiency for nitrogen fixation. El-Fiki (2006) reported that RAPD finger printing were used for strain identification the assessment of genetic diversity with in a field population of *Rhizobium*.

5.5. Carbohydrate utilization by *Rhizobium*

Among 31 isolates, four isolates were selected on the basis of their growth behaviour Rh01 and Rh13 (*Vigna mungo*) Rh05 and Rh09 (*Arachis hypogaea*) were selected and isolates VRS1 and VRS2 from *Vigna mungo* and ARS1 and ARS2 from *Arachis hypogaea* root nodules.

The present observation, four isolates were able to grow well in the presence of glucose, galactose, maltose, xylose and fructose. ARS1 *Rhizobium* strains were utilized all carbohydrates as a carbon source. Arabinose, Sucrose was not utilized by VRS1 isolates. Kucuk *et al*. (2006) have proposed that, the Eskisehir isolates were able utilize the several compounds as sole sources of carbon. All isolates were able to grow well in the presence of D(-) fructose, D(+) galactose, D(+) glucose, D(+) mannitol, sucrose, starch, succinate and rhamnose. Bean rhizobial isolates utilized a wide range of carbohydrates and salts of organic acids as carbon sources (Hungria and Vargas, 2000).
In the present investigation, all four strains showed good growth on carbohydrates. Similar observations were also reported in *Rhizobium* isolates from root nodules of *Sesbania sesban* (Helmish *et al*., 1993). From the present study it is evident that all the strains effectively utilized a wide range of carbohydrates, it is one of the important criteria to be considered as plant growth promoting bacteria.

**5.6. Effect of pH on the growth of *Rhizobium***

Soil acidity is a significant problem facing agricultural production in many areas of the world and limits legume productivity. pH is an important parameters for the growth of the *Rhizobium*. Slight variations in pH of medium might have enormous effects on the growth of organism. *Rhizobium* has been reported to grow the best at neutral pH i.e., 7.

In the present observation, pH 7 recorded maximum absorbance for the broth experiment as well as exhibited heavy growth as measured by the population count on the one week after incubation of VRS1 and VRS2 rhizobial isolates. Mensah *et al.* (2006) recorded that the optimum pH of the growth of *Rhizobium* is 7. Similar findings were made by Singh *et al.* (2008) reported that *Rhizobium* were able to grow at pH 7 and kept at 29.4°C but no growth was observed in medium with pH 4 and 9.

ARS1 and ARS2 isolates were showed good growth between pH 5 and 8, but less growth was observed at pH 3, 4 and 9. These observations are in line with the reports of Aurag and Sasson (1992) indicated that strains of *Rhizobium leguminosarum* bv. *phaseoli* grew in liquid media of pH 5. In the present study, all rhizobial isolates growth rate was increased at all the pH level on II and III week of growth. ARS1 and ARS2 isolates
was tolerant to pH 5 and 8. Rhizobia with a higher tolerance to acidity and alkalinity will be of great impacts in acidic or alkaline soil conditions in the field.

5.7. Effect of temperature on the growth of *Rhizobium*

Temperature is one of the most important factor affecting the survival of rhizobia in soil. High temperature is an essential ecological factor in Tamil Nadu agricultural fields. The rhizobia capable to survive and grow under these conditions are very important for inoculants production. Graham (1992) reported that, for most rhizobia the optimum temperature range for growth in culture is 28 to 31°C and many are unable to growth at 37°C.

In the present investigation, optimum growth temperature were 35°C for VRS1, VRS2, ARS1 and ARS2 strains. The growth of all the strains decreased gradually low (20°C) and high (40°C) temperatures. The four rhizobial isolates were able to increase growth rate at I and II week whereas at III week growth rate was decreased. Kucuk *et al.* (2006) reported that the among the 30 isolates, 18 were able to grow at 37 and 40°C, whereas 5 isolates showed only minimal growth at 42 and 45°C. In the present study, all these 4 strains were tolerant to temperature of 35°C, selection of these effective strains of *Rhizobium* to inoculate legumes in Tamil Nadu agricultural field or soils will be of great impacts in such high temperature conditions.

5.8. Effect of NaCl concentration on the growth of *Rhizobium*

Salt is a serious threat to agricultural lands in Tamil Nadu. High salt concentrations may have a detrimental effect on soil microbial populations. The root nodule-colonizing bacteria (*Rhizobium*) are more
salt tolerant than their legume hosts, they show marked variation in salt tolerance. Embalomatis et al. (1994) reported that the growth of a number of rhizobia was inhibited by 100 mM NaCl, while some rhizobia, e.g., *Rhizobium meliloti* were tolerant to 300-700 mM NaCl.

The results of the present studies revealed that salt concentration in the growth medium had significant effect on the growth of *Rhizobium*. VRS1, VRS2, ARS1 and ARS2 strains grew lightly at 0.2 M NaCl, but the growth was heavy at lower salt concentrations. Previous studies reported by Talibart et al. (1994) have shown that the changes in osmotic potential exerted by salt concentration alter the structure of lipopolysaccharides of bacteria in response to salt stress and that rhizobia accumulate several solutes to overcome the osmotic stress induced by salt when growing in association with the host plant.

Lloret et al. (1995) also reported that the *Rhizobium* strains capable of growing at NaCl concentrations of upto 0.5 M have been isolated from melilotus plants. In the present study, all rhizobial isolates were able to grow on 0.00625 – 0.15 M NaCl and growth rate was increased from I week to II week whereas in III week, growth was decreased for both isolates. This similar findings were recorded by Mensah et al. (2006).

**5.9. Antibiotic resistance pattern for *Rhizobium***

Antibiotic resistance in *Rhizobium* strains was tested against different antibiotics. VRS1, VRS2, ARS1 and ARS2 rhizobial isolates were resistance to penicillin and cefazolin and moderate resistance to ampicillin, methicillin and Bacitracin antibiotic. But, all there four strains were highly sensitive to tetracycline antibiotics.
Issa and Wood (1995) reported that the use of relatively low (or) moderate concentration of the antibiotics give a more reliable information about the IAR and how far is the range of resisted antibiotics a *Rhizobium* strain can tolerate and this could be very helpful on the ecological and diversity studies of rhizobia.

In general, data obtained from this study clearly show that certain concentration of antibiotics used in the medium (YEMA) was suitable for differentiation between members of the rhizobial groups. Depends on the differences between strains of rhizobia towards the different antibiotics, this technology could be successfully, employed for the field of ecological studies of rhizobia. This similar observations were reported by Milcic *et al.* (2006).

**5.10. Protein studies in *Rhizobium***

The maximum amount of protein content 90.625 mg/ml was recorded from VRS1 *Rhizobium* strain followed by VRS2 78.125 mg/ml, ARS2 71.875 mg/ml and ARS1 39.0625 mg/ml.

In the present study, electrophoretic banding pattern of cell protein of four *Rhizobium* isolates was performed, to find out protein profile by SDS-PAGE electrophoresis. SDS-PAGE of whole cell proteins of rhizobial strains from the wild legumes, exhibited protein profiles with peptide bands ranging from 5-19 bands per profile was reported by Zahran *et al.* (1994). Fabiano and Arias (1990) reported that the SDS-PAGE analysis of whole cell proteins not only helps in identifying of the rhizobial strains but also useful in the differentiation among the isolates within the same sero group.
The total cell protein profiles thus suggests that the patterns are isolate-specific and can serve as fingerprints, it can hence be concluded that chickpea-rhizobia isolated from same soil-climatic region are diverse (Khokhar et al., 2001).

5.11. DNA studies in *Rhizobium*

DNA was estimated by diphenylamine method and plasmid DNA was determined by Agarose gel electrophoresis. The maximum amount of DNA was estimated from the VRS1 followed by ARS2, ARS1 and VRS2 *Rhizobium* strains. DNA content of cell is used for cell multiplication, but some environmental stresses may be inhibit the DNA synthesis. Johnson and Wood (1990) reported that when Aluminium was taken up by the Rhizobia, Al was bound to the DNA of both sensitive and tolerant strains but that DNA synthesis was not affected in tolerant strains of *Rhizobium loti*.

The plasmid DNA of VRS1, VRS2, ARS1 and ARS2 shows high plasmid diversity. Bromfield et al (1995) examined the diversity of plasmid content among field populatons of *Rhizobium meliloti* and on the plasmids role in nitrogen fixation is a very important concept for future research.

Plasmids may be carried some functions related to the *Rhizobium* infections process on legumes, life in the rhizosphere and life in the soil. Genes are essential for the symbiotic relationship with legumes. Weaver (1990) found that there are differences in growth among *Rhizobium phaseoli* bearing different types of plasmids and it has been reported that plasmids can influence the growth of rhizobia in liquid cultures.
5.12. Nodulation ability of *Rhizobium*

VRS1 and ARS2 rhizobial isolates were selected and used in nodulation ability tests. VRS1 and ARS2 strains were formed nodules in *Vigna mungo* and *Arachis hypogaea* respectively. Both these isolates showed abundant nodulation, whereas nodules were decreased in uninoculated control plants. The number of nodules, nodule fresh weight and plant dry weight were increased by VRS1 and ARS2 *Rhizobium* isolates in all nodulation ability tests such as, test tube method new nodulation ability test, plastic cup method and Green house experiment.

Mahmood and Athar (2008) have stated that the highest number of nodules were produced by Rhizobial isolates in *Vigna mungo*. Several reports describe in successful experiments where wild rhizobia are more effective in nitrogen fixation than their compatible host (Zahran, 2001).

Inoculation with *Rhizobium* strains induced significant increase in nodules numbers were recorded in roots of alfalfa plants (Hegoo and Barakah, 2004) and also stated that, if soil is free from *Rhizobium meliloti* no nodules were formed in uninoculated controls. From this investigation indicated that the nodulation of agriculturally important legumes with rhizobial isolates may prove a useful means of increasing nitrogen contents within these plants.

5.13. Effect of salt stress on growth of plants

Low salt levels reduced the plant growth and nodule number to about 50 per cent of the non-salt treatment. High salt levels decreased the nodule number and weight to about 35 and 30 per cent in *Arachis hypogaea* and *Vigna mungo* plants respectively, compared to the control
treatment. The results in present investigation indicate that both plants inoculated with *Rhizobium* strain more tolerant to salt stress than uninoculated plants.

Comparing the present study with previous findings of Wahab *et al.* (2002) reported that the nodulation in *Lablab purpureus* (L.) was almost completely suppressed at a salinity 120 mM NaCl compared to 35 per cent of controls. In this study, shoot and root length also affected at high salinity levels of both plants. But inoculated stressed plants showed increase level of morphological changes and nodulation than control stressed plants. It has been reported by Diejomaoh (1996) that morphological changes including reduced plant height and yield are mainly due to the effect of the salt on the plant rather than changes due to the symbiotic relationship with the *Rhizobium*. Furthermore, he stated that the reductions in plant height and dry weight biomass are as a result of decreases in the size of the individual cells/structures.

In this present study, *Arachis hypogaea* and *Vigna mungo* plants root and shoot length, leaves length and width, nodule number and nodule fresh and dry weights showed significant decreases with increasing salt concentrations. This similar findings were reported by Taffouo *et al.* (2010) in *Arachis hypogaea* (L.) cultivars under salt stress. Tejera *et al.* (2004) reported that shoot growth was affected by salt than the roots growth in beans.

In this investigation, morphological changes and nodulation was adversely affected in control stressed plants than inoculated plants. The negative action of salt stress on nitrogen fixation may be due to three
different responses: effect on the infection of legumes by rhizobia, effect on nodule growth and development and finally, direct effect on nodules activity. Zahran (1999) reported that the several symbiotic systems of legumes which are tolerant to extreme conditions of salinity, acidity, drought, etc.

5.14. Effect of water stress on growth of plants

In leguminous plants, drought reduces nitrogen fixation and its related traits. The leguminous plants inoculated with *Rhizobium* strain moderately resistant to drought stress than the uninoculated plants. The use of drought resistant cultivars should be of great advantage to retain high nitrogen fixation and acceptable yield. Drought is one of the major factor to reduced the growth and yield components of field crops.

The water stress decreasing the nodulation and plant growth parameters. However, the presence of more than 50 per cent active nodules indicates that our experimental plants, *Arachis hypogaea* and *V. mungo* are tolerant to mild water deficits. The nodules of *Rhizobium* inoculated plants yielded the greatest weights.

Similar findings were made by Figueiredo et al. (1998) reported that the water-stress effect on different stages of N₂ fixation in cowpea plants. Drought stress reduced nodule number and nodule fresh and dry weights, but the reductions in these traits differed among traits and between levels of drought stress. The results indicated that levels of stress had significant effects on the *A. hypogaea* and *V. mungo* plants. Similar findings were reported by Pimratch et al. (2008). Several mechanisms have been suggested and reviewed to explain the varied physiological
responses of several legumes when subjected to drought (Ramos et al., 1999).

Soil moisture effect on N₂-fixation, shoot and root metabolism, nodule number and weights of nodule. Wade et al. (2006) found that the water stress effects on root dry matter, root length, nodule number and nodule dry weight in cowpea plants. In addition, Irogoyen et al. (1992) noticed that the water deficits directly affect metabolic process in nodules and induce changes in the concentration of proline and sugar in alfalfa nodules.

The local cultivars *Arachis hypogaea* and *V. mungo* plants is more tolerant to water stress when inoculated with *Rhizobium* than the uninoculated plants. Moreover, the results obtained here indicate that *Rhizobium* is more suitable for cultivation of *A. hypogaea* and *V. mungo* plants.

### 5.15. Estimation of nitrogenase activity

The measurement of nitrogenase activity was based on the reduction of acetylene to ethylene as quantities by gas chromatography. Nitrogenase activity of both plant nodule inoculated with rhizobial isolates showed maximum acetylene reduction than uninoculated stressed plants. Pimratch et al. (2008) reported that the contributions of potential and reduction in the nitrogenase activity were different between drought stress levels. The contribution of potential was high under mild drought and it was reduced with more severe stress. Georgiev and Atkins (1993) reported that nitrogenase activity of cowpea root nodules was depressed by NaCl.
In the present study, nitrogenase activity of both plants were affected by salt and water stress. Ramos et al. (1999) reported that nodule number, weight and ARA of Phaseolus vulgaris cultivars were significantly reduced at 50 per cent soil FC. Nodulation and nitrogenase activity in both plant roots were responded greatly to inoculation of Rhizobium. The present study clearly showed that the leguminous plants, if free from Rhizobium, nodules and nitrogenase activity was decreased and also it values tend to increase significantly by increasing plant growth.

5.16. Determination of leghaemoglobin content

The presence of leghaemoglobin in legume nodules is considered as a prerequisite for N\textsubscript{2}-fixation. Leghemoglobin is an nitrogen or oxygen carrier, it has a high affinity for oxygen and allows nitrogenase to function during N\textsubscript{2}-fixation.

In the present investigation, the highest LHB was recorded with plants that were given the basal nutrient solution without any added salt (controls). Values of leghaemoglobin content slightly decreased at high salinity levels. Also LHB considerably declined with decrease in water potential. The tendency of LHB content was to increase with plant growth (55 DAT). Swaraj et al. (1995) reported that the increase in the severity and duration of water stress affected the nitrogenase activity and leghaemoglobin content of nodules of chickpea plants. Delago et al. (1994) found no direct relationship between the effect of salinity on the LHB to soluble protein ratios and ARA in V. faba and Phaseolus nodules.

From the present investigation, it may be concluded that the rhizobial isolates showed better symbiotic performance with leguminous
plants. Several environmental conditions are limiting factors to the growth and activity of this N\textsubscript{2}-fixing plants. In the *Rhizobium*-legume symbiosis, the process of N\textsubscript{2} fixation is strongly related to the physiological state of the host plant. Therefore, a competitive and persistent *rhizobial* strain is not expected to express its full capacity for nitrogen fixation if limiting factors (e.g., salinity, unfavourable soil pH and temperature extremes) impose limitations on the vigour of the host legume. The *Rhizobial* isolates showed tolerant to salt and drought stress than the control and fix high level of N.