EFFECTS OF SALINITY ON THE NODULATION IN LENTIL (*Lens culinaris* Medik.) AND THE DIVERSITY OF ITS COMPATIBLE NITROGEN-FIXING *Rhizobium*

A THESIS
SUBMITTED IN PARTIAL FULFILLMENT FOR THE DEGREE OF MASTER OF PHILOSOPHY IN BOTANY, UNIVERSITY OF DHAKA

SUBMITTED BY
JANNATUL FERDOUS
SESSION: 2013-2014
REG. NO.: 195
EXAM ROLL: 03

ECOLOGY AND ENVIRONMENT LABORATORY
DEPARTMENT OF BOTANY
UNIVERSITY OF DHAKA
DHAKA-1000, BANGLADESH

DECEMBER 2018

DEDICATED

TO

MY FAMILY AND TEACHERS

CERTIFICATE

This is to certify that the research work embodying the result of the thesis entitled **"Effects of salinity on the nodulation in Lentil (*Lens culinaris* Medik.) and the diversity of its compatible nitrogen-fixing *Rhizobium*"** by **Jannatul Ferdous** has been carried out at the Plant Ecology and Environment Laboratory, Department of Botany, University of Dhaka, under our supervision and the style and contents of this thesis is suitable for the partial fulfillment of the Degree of Master of Philosophy in Botany.

Supervisor
Mohammad Zabed Hossain (Ph.D.)
Professor
Ecology and Environment Laboratory
Department of Botany
University of Dhaka
Dhaka 1000
Bangladesh

Co-Supervisor
Ashfaque Ahmed
Professor
Ecology and Environment Laboratory
Department of Botany
University of Dhaka
Dhaka 1000
Bangladesh

ACKNOWLEDGEMENT

Having completed this study, I am grateful to my supervisor Mohammad Zabed Hossain (Ph.D., UGAS, Iwate University, Japan; Post-Doctorate, SLU, Uppsala, Sweden), Professor, Department of Botany, University of Dhaka, for his constant support, guidance, supervision, encouragement and cooperation during the time of my research work. I am also thankful to my co-supervisor Ashfaque Ahmed, Professor, Department of Botany, University of Dhaka for his valuable suggestion and endless encouragement during the period of my research.

I feel proud to express my sense of gratitude to the Chairman, Professor Dr. Rakha Hari Sarker and at the same time to the former Professor Chairman Moniruzzaman Khondker and Professor Dr. M A Bashar of the Department of Botany, University of Dhaka for providing all laboratory facilities during the study period.

I would like to send my love to my lab mates Nur Jahan apu, Habib bhai, Ataur bhai, Moshidul bhai, Protika didi, Shaila, Rasel, Nabila, Jake, Pranty, Pragga, Sydul and Suraiya for their co-operation and encouragement. Specially I am thankful to Kashem, Mehedi and Tropa who stood behind me whenever I needed. They should be aware of that they will not be forgotten ever.

I would like to remember my mother late Zotsona Begum. I am thankful to my father, my brothers and my sister in laws for their strong encouragement, support and inspiration. I am also thankful to my in laws family for their understanding and endless love. Specially I am grateful and would like to express my heartfelt gratitude to my husband Nazrul Islam for his encouragement and support.

Finally, I express my heartiest gratitude to Almighty Allah who has enabled me to complete this work.

ABSTRACT

Salinity in soil causes risk for growth, nodulation and biological nitrogen fixation in legumes. Biological nitrogen fixation occurs through the symbiotic association between legumes and *Rhizobium*. Legume-rhizobia symbiosis and the efficiency of biological nitrogen fixation are influenced by a number of environmental factors including salinity. This study was carried out to evaluate the effects of salinity on the growth parameters and nodulation of lentil plants (*Lens culinaris* Medik.) at various NaCl concentrations. Seeds of lentil were sown in the experimental plots at the Botanical Garden of University of Dhaka. Each of the four salt treatments (0 mM, 50 mM, 100 mM and 200 mM NaCl) was applied in three replicate plots. Treatments were applied once in every seven days. The size of each plot was 0.0625 m^2. Plants were grown for 2 months in the field. Shoot and root growth parameters including length, fresh and dry weight of shoot and root, leaf chlorophyll content, root to shoot ratio, root nodule number and proline content of leaf were compared among the different salt treatments. This study also examined the effects of soil salinity on the number of bacterial colonies obtained from root nodules, rhizosphere soil and bulk soil of lentil plants. The suspensions of root nodules were cultured in the nutrient agar medium and then bacterial colonies were isolated and counted. Bacterial DNA were isolated from pure isolates of bacterial colonies obtained from soils collected from different areas of Bangladesh. PCR amplification using bacterial DNA samples as template was done by targeting 16s rRNA gene. After purified PCR product was achieved, the sequences of different strains of rhizobia were identified by using NCBI blast. Phylogenetic tree was also constructed based on the 16S rRNA sequencing of isolated bacteria. In comparison to control (0 mM), shoot height, root fresh

weight, number of nodules and chlorophyll content of leaf decreased with the increase of salt treatment although no significant difference appeared. Proline concentration of leaf of lentil increased significantly with the increase of salt concentrations. Number of bacterial colony increased with the increase of salt concentration. Compared to bulk soil, rhizosphere soil showed higher number of bacterial colonies. The result thus indicates that soil salinity can influence bacteria associated with the root nodule and rhizosphere of lentil plants. The clusters of phylogenetic tree indicated the genetic diversity of rhizobia associated with root nodules collected from different sites. Result of this study is expected to provide valuable information and may be useful to the researchers as well as the policy makers of both Government organizations and Non-government organizations for formulating appropriate policy for widespread cultivation of lentil in Bangladesh.

CONTENTS

1. GENERAL INTRODUCTION 1
1.1 Global scenario of soil salinity 1
1.2 Effects of soil salinity on plants 2
1.3 Soil salinity status in coastal areas of Bangladesh 3
1.4 Impact of salinity on agriculture of Bangladesh 5
1.5 Mechanisms of salinity- induced effects on plants 7
1.6 Lentil – a nutrient rich crop 9
1.7 Ecological requirements of lentil plants 11
1.8 Lentil cultivation in Bangladesh 11
1.9 Role of legumes in soil nitrogen (N) availability 14
1.10 Symbiotic association between legume and rhizobia 15
1.11 Nodule formation by nodulation genes and *nod* factors 17
1.12 Taxonomy of *Rhizobium* 19
1.13 Characteristics of rhizobial bacteria 20
1.14 Significance of biological nitrogen fixation for soil fertility 21
1.15 Effects of salinity on biological nitrogen fixation 24
1.16 Molecular markers used to study genetic diversity of *Rhizobium* 26
1.17 Objectives of the research work 30
2. MATERIALS AND METHODS 31
2.1 Effects of salinity on the growth and development of lentil plants 31
2.1.1 Seed selection and surface sterilization 31
2.1.2 Salt treatment 31
2.1.3 Experimental plot preparation 32
2.1.4 Growing plants 32
2.1.5 Determination of shoot height 34
2.1.6 Measurement of root length 34
2.1.7 Determination of shoot fresh and dry biomass 34
2.1.8 Determination of root fresh and dry biomass 35

2.1.9 Determination of leaf chlorophyll content 35
2.1.10 Extraction and determination of proline 35
2.1.11 Collection of root nodule 36
2.1.13 Data analysis 36
2.2 Effects of salinity on the bacterial population 37
2.2.1 Collection of root nodule 37
2.2.2 Collection of rhizosphere soil and bulk soil from the field 41
2.2.3 Surface sterilization of the root nodules 41
2.2.4 Isolation of bacterial cells from nodule 42
2.2.5 Preserving the root nodules 42
2.2.6 Preparation of Congo red-YMA medium (for 500 ml) 42
2.2.7.1 Procedure for preparation of the medium 43
2.2.7.2 Requirements for plating 43
2.2.7.3 Plating of the bacterial suspension in the medium 44
2.2.7.4 Maintenance of the cultures 44
2.2.8 Morphological colony characteristics of the isolates 44
2.2.9 Data analysis 44
2.3 Identity and genetic diversity of rhizobia 45
2.3.1 Requirements for bacterial DNA isolation 45
2.3.2 Extraction of DNA from bacterial colony 46
2.3.3 Estimation of quality and quantity of isolated DNA samples 46
2.3.4 Requirements for PCR 46
2.3.5 Primers used 47
2.3.6 Preparation of working concentrations of DNA samples for PCR reaction 47
2.3.7 Preparation of primer 47
2.3.8 Preparation of PCR reaction mixture 48
2.3.9 PCR conditions 49
2.3.10 Polymerase Chain Reaction (PCR) 49
2.3.11 Requirements for Agarose gel electrophoresis 49
2.3.12 Preparation of stock solutions used for Gel–electrophoresis 50
2.3.12.1 ò TAE buffer (p^H 8.3) (1 liter) 50

2.3.12.2 6òLoading dye 50
2.3.12.3 Ethidium bromide solution 51
2.3.13 Agarose Gel-electrophoresis 51
2.3.14 Agarose Gel electrophoresis of the amplified products 52
2.3.15 Documentation of the DNA sample 52
2.3.16 Purification of PCR product 52
2.3.17 Determination of the concentration of purified PCR product 53
2.3.18 Sequencing and identification of bacteria 53
2.3.19 Data analysis 54
3. RESULTS 55
3.1 Effects of salinity on the growth and development of lentil plants 55
3.1.1 Effects of salinity on shoot growth parameters 55
3.1.2 Effects of salinity on root growth parameters 56
3.1.3 Proline 57
3.2 Effects of salinity on bacterial the population associated with roots of lentil 68
3.2.1 Effects of salt concentrations on the bacterial colonies in root nodule 68
3.2.2 Number and size of bacterial colonies obtained from rhizosphere and bulk soil 70
3.3 Identity and genetic diversity of *Rhizobium* 80
4. DISCUSSION 86
5. REFERENCES 93
APPENDIX i

LIST OF FIGURES

Figure 1.1. Saline zones of Bangladesh (Source: Soil Resource Development Institute, 2009) 5
Figure 1.2. Lentil cultivated areas in Bangladesh. 14
Figure 1.3. Process of root nodule formation in legume plants. 18
Figure 1.4. Structure of 16s ribosomal RNA short subunit. 29
Figure 2.1. Experimental field at the Botanical Garden at Curzon Hall area University of Dhaka. After 21 days (a and c) and after 14 days (b). 34
Figure 2.2. Sampling sites (round symbol) selected for the collection of lentil plants used in the present study. 38
Figure 2.3. Sites selected for the collection of root nodule: Botanical Garden at Curzon Hall area, Univesity of Dhaka (a); lentil field in Cumilla (b); lentil field in Rajshahi (c).39
Figure 2.4. Root nodules collected from the lentil plant grown under different concentrations of salt treatment: 50 mM NaCl (a1, a2 and a3); 100 mM NaCl (b1, b2 and b3); 200 mM NaCl (c1, c2 and c3). 40
Figure 3.1. Shoot height (mean ± SEM) of *Lens culinaris* Medik. var. BARI masur-4 plants treated with varying concentrations (0 mM, 50 mM,100 mM and 200 mM) of NaCl. 58
Figure 3.2. Shoot fresh weight (mean ± SEM) of *Lens culinaris* Medik.var. BARI masur-4 plants treated with varying concentrations (0 mM, 50 mM, 100 mM and 200 mM) of NaCl. 59
Figure 3.3. Shoot dry weight (mean ± SEM) of *Lens culinaris* Medik. var. BARI masur-4 plants treated with varying concentrations (0 mM, 50 mM,100 mM and 200 mM) of NaCl. 60
Figure 3.4. Chlorophyll content (mean ± SEM) of *Lens culinaris* Medik.var. BARI masur-4 plants treated with varying concentrations (0 mM, 50 mM,100 mM and 200 mM) of NaCl. 61

Figure 3.5. Root length (mean ± SEM) of *Lens culinaris* Medik. var. BARI masur-4 plants treated with varying concentrations (0 mM, 50 mM,100 mM and 200 mM) of NaCl. 62

Figure 3.6. Root fresh weight (mean ± SEM) of *Lens culinaris* Medik. var. BARI masur-4 plants treated with varying concentrations (0 mM, 50 mM,100 mM and 200 mM) of NaCl. 63

Fig. 3.7. Root dry weight (mean ± SEM) of *Lens culinaris* Medik. var. BARI masur-4 plants treated with varying concentrations (0 mM, 50 mM,100 mM and 200 mM) of NaCl. 64

Figure 3.8. Root:Shoot (mean ± SEM) of *Lens culinaris* Medik. var. BARI masur-4 plants treated with varying concentrations (0 mM, 50 mM,100 mM and 200 mM) of NaCl. 65

Figure 3.9. Nodule number per plant (mean ± SEM) of *Lens culinaris* Medik.var. BARI masur-4 plants treated with varying concentrations (0 mM, 50 mM,100 mM and 200 mM) of NaCl. 66

Figure 3.10. Proline content (mean ± SEM) of *Lens culinaris* Medik.var. BARI masur-4 plants treated with varying concentrations (0 mM, 50 mM,100 mM and 200 mM) of NaCl. 67

Figure 3.11. Plates showing bacterial colony cultured from root nodule of lentil plants treated with varying concentrations (0 mM, 50 mM, 100 mM and 200 mM) of NaCl. ... 69

Figure 3.12. Number of small bacterial colony (mean ± SEM) cultured from root nodule of Lens culinaris Medik.var. BARI masur-4 plants treated with varying concentrations (0 mM, 50 mM,100 mM and 200 mM) of NaCl. 74

Figure 3.13. Number of medium bacterial colony (mean ± SEM) cultured from root nodule of Lens culinaris Medik.var. BARI masur-4 plants treated with varying concentrations (0 mM, 50 mM,100 mM and 200 mM) of NaCl. 75

Figure 3.14. Number of large bacterial colony (mean ± SEM) cultured from root nodule of Lens culinaris Medik.var. BARI masur-4 plants treated with varying concentrations (0 mM, 50 mM, 100 mM and 200 mM) of NaCl. 76

Figure 3.15. Number of small bacterial colony (mean ± SEM) cultured from rhizosphere soil and bulk soil under Lens culinaris Medik.var. BARI masur-4 treated with varying concentrations (0 mM, 50 mM,100 mM and 200 mM) of NaCl. 77
Figure 3.16. Number of medium bacterial colony (mean ± SEM) cultured from rhizosphere soil and bulk soil under Lens culinaris Medik.var. BARI masur-4 treated with varying concentrations (0 mM, 50 mM, 100 mM and 200 mM) of NaCl. 78
Figure 3.17. Number of large bacterial colony (mean ± SEM) cultured from rhizosphere soil and bulk soil under Lens culinaris Medik.var. BARI masur-4 treated with varying concentrations (0 mM, 50 mM, 100 mM and 200 mM) of NaCl. 79
Figure 3.18. Picture showing Gel electrophoresis. R1, R2, R3, R4, R5, R6 obtained from root nodule of Rajshahi area and C8, C9, C10, C11, C12 obtained from root nodule of Cumilla area. 81

LIST OF TABLES

Table 1. The area and production of lentil in Bangladesh from 2009-10 to 2015-2016 (BBS 2016). 12

Table 3.1. Mean values with standard error mean of the number of different colonies (Small colony, medium colony and large colony) from rhizosphere soil and bulk soil at different concentrations of NaCl (0 mM, 50 mM, 100 mM and 200 mM) after 24 hours. 72

Table 3.2. Two-way ANOVA statistics on the effects of rhizosphere soil and bulk soil on different sizes of bacterial colonies (Small colony, medium colony and large colony) after 24 hours of incubation. 73

Table 3.3. Estimation of nucleic acid concentrations (ng/µl) of the bacterial isolates. 80

Table 3.4. Bioinformatics of the DNA sequences obtained from rhizobium isolates using NCBI (National Centre for Biotechnology Information) BLAST. 84

1. GENERAL INTRODUCTION

1.1 Global scenario of soil salinity

Salinity in soil has created enormous concern among the people worldwide. Soil salinity becomes a major issue in global agriculture. The progressive salinization of land is one of the most severe problems in agricultural production worldwide (Greenaway and Munns 1980), limiting plant growth and productivity of the arid or semiarid environments. Globally, more than 800 million hectares of land are estimated to be salt affected (FAO 2008). These soils cover a range of soils defined as saline, saline-sodic and sodic (Ghassemi *et al.* 1995). All soil types with diverse morphological, physical, chemical and biological properties may be affected by salinity (Rengasamy 2006).

A major challenge towards world agriculture involves production of 70% more food crop for an additional 2.3 billion people by 2050 (FAO 2009). Salinity is a major limiting factor that increases the demand for food crops. More than 20% of cultivated land worldwide (~ about 45 million hectares) is affected by salt stress and the amount is increasing day by day (FAO 2009).

Worldwide, more than 45 million hectares of irrigated land have been damaged by salt, and 1.5 million hectares are taken out of production every year as a result of high salinity levels in the soil (Munns and Tester 2008). High salinity affects plants in several ways. Together, these effects reduce plant growth, development and survival (Hasegawa *et al.*

2000). Global climate change has further accelerated the salinity problems by increasing rise of sea level as consequences of global warming and melting of polar ice.

1.2 Effects of soil salinity on plants

Soluble salt present in excessive concentrations in the soil is known as soil salinity. The process of increasing the salt content in the soil is known as salinization. All soils contain some water soluble salts. Plants absorb essential plant nutrient in the form of soluble salts, but excessive accumulation of soluble salts causes salinity. Soil can be saline due to geo-historical processes or they can be man-made. Salinity can be caused by natural processes such as mineral weathering or by the gradual withdrawal of an ocean. It can also occur through anthropogenic processes such as irrigation. Salt affected soils are common in coastal area in tropical regions, arid and semi-arid regions. Salt stress is one of the most serious limiting factors for crop growth, development and production in the arid regions. About 23% of the world's cultivated land is saline and 37% is sodic (Khan and Duke 2001). Salinity and soil nutrient deficiencies are the main factors which reduce plant production in the semi-arid and arid areas around the world (McWilliam 1986, Shannon *et al.* 1994).

Soil salinity in agricultural soils refers to the presence of high concentration of soluble salts in the soil moisture of the root zone. These concentrations of soluble salts affect plant growth by restricting the uptake of water by the roots. The major cations contributing to salinity are Na^+, Ca^{2+}, Mg^{2+}, K^+ and anions are Cl^-, SO_4^{2-} HCO^{3-}, CO_3^{2-} and NO_3^{2-}. There exist also trace ions including B, Sr, Li, Rb, F, Mo, Ba, and Al (Tanji

1990). However, salinity is used to mean Na^+ concentration of the growth solution, which is the main constituent of the saline soils. Salinity can also affect plant growth because the high concentration of salts in the soil solution interferes with nutritional balance. NaCl toxicity, the predominant form of salt in most saline soils, enhances the sodium content and consequently affects the absorption of other mineral elements (Greenway and Munns 1980). Indeed, high levels of Na inhibit Ca and K absorption, which results in a Na/K antagonism (Benlloch *et al.*1994). On the other hand, the maintenance of high tissue K/Na ratio as criteria for salt-tolerance is also affected (Ashraf and McNeilly 2004). Salinity inhibits the growth of plants, through reduced water absorption, reduced metabolic activities due to Na^+ and Cl^- toxicity, and nutrient deficiency caused by ionic interference (Yeo 1983). Salinity influences the productivity of agricultural crops, with adverse effects on plant growth parameter (Munns and Tester 2008). Salinity affects many irrigated areas mainly due to the use of brackish water.

1.3 Soil salinity status in coastal areas of Bangladesh

There are various causes which have been anticipated for increasing soil salinity. Among the various causes withdrawal of fresh river water from upstream, irregular rainfall, introduction of brakish water for shrimp cultivation, faulty management of the sluice gates, regular saline tidal water flooding in unprotected area and capillary rise of soluble salts are some of the main causes of increasing soil salinity in the topsoil's of the coastal region (Hossain *et al.* 2016b).

Out of 1.689 million hectares of coastal land, 1.056 million hectares are affected by soil salinity of various degrees. About 0.328, 0.274, 0.189, 0.161 and 0.101 million hectares of land are affected by very slight, slight, moderate, strong and very strong salinity, respectively. These lands cover about one fifth of the total areas of Bangladesh. Some of the new land of Satkhira, Patuakhali, Borguna, Barisal, Jhalakathi, Pirojpur, Jessore, Narail, Gopalgong and Madaripur districts are affected by different degrees of salinity, which reduces agricultural productivity remarkably (Ashraf and Waheed 1990). Distribution of soils with different degrees of salinity is shown on Soil Salinity map (Fig. 1.1). A comparative study of soil salinity during last four decades (1973-2009) in coastal areas showed that about 50% of the coastal lands face different degrees of inundation, thus limiting their effective use. This situation is expected to become worse further because of the effects of climate change (SRDI 2010).

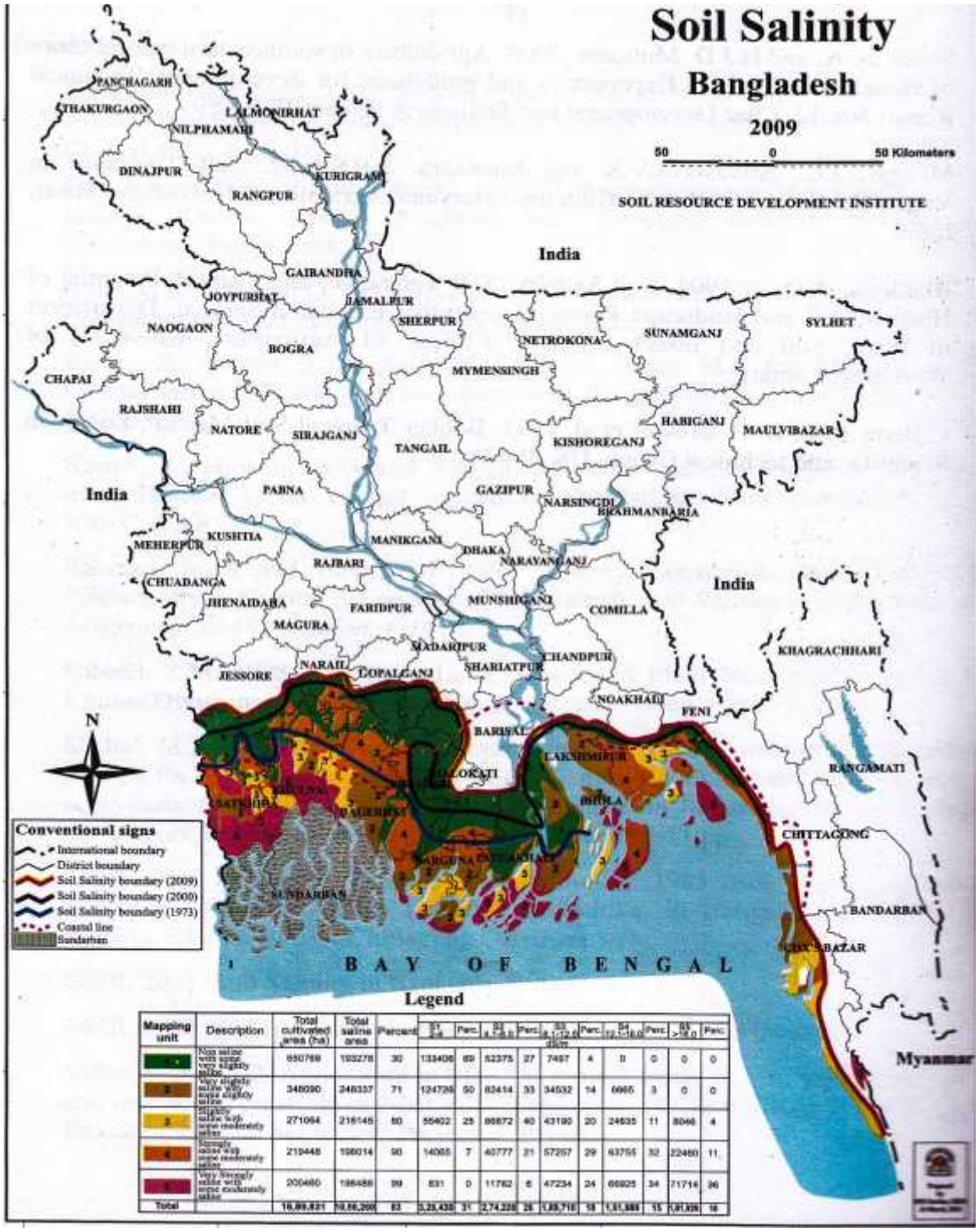

Fig. 1.1. Saline zones of Bangladesh (Source: Soil Resource Development Institute, 2009).

1.4 Impact of salinity on agriculture of Bangladesh

The coastal area covers about 20% of the total area of Bangladesh which is 147,570 km^2. It extends inside up to150 km from the coast. Out of 2.85 million hectares of the coastal and offshore areas, about 0.83 million hectares are arable lands, which cover over 30% of the total cultivable lands of Bangladesh. A part of the coastal area, the Sundarbans, is a reserve natural mangrove forest covering about 4,500 km^2. The remaining part of the coastal area is used in agricultural purpose. The cultivable areas in coastal districts are affected with varying degrees of soil and water salinity. The coastal and offshore area of Bangladesh includes tidal, estuaries and river floodplains in the south along the Bay of Bengal. Agricultural land use in these areas is very poor, which is roughly 50% of the country's average. Salinity causes unfavorable environment and hydrological situation that restrict normal crop production throughout the year (Islam 2003). Soil salinity has emerged as a problem which is not only reducing the agricultural productivity (Ali 2006, Battacharya *et al*. 1999, Singh and Singh 1995) but also putting far reaching impacts on livelihood strategies of farmers. Both of the soil and water salinity have adverse effects on people's living standard, daily life activities and socio-economic conditions (Haque and Saifuzzaman 2003, Miah *et al.* 2004, Tanwir *et al.* 2003). People in the southwest region are highly dependent on the natural resource for sustaining their livelihoods. Agriculture and fishery are important economic sectors, employing a large proportion of the population, and aquaculture is increasingly being pursued as an alternative livelihood option for rural households (Islam 2003). The region is densely populated, and most farm families cultivate the scarce land resources intensively, resulting in land degradation and reduced productivity. This situation is compounded by increasing salinity and water

logging which further reduces potential cultivating land by increasing salinity and water logging, further reducing the availability of cultivable land.

1.5 Mechanisms of salinity- induced effects on plants

Salinity stress involves changes in various physiological and metabolic processes, depending on severity and duration of the stress, and ultimately inhibits crop production (James *et al.* 2011, Rahnama *et al.* 2010, Munns 2005, Rozema and Flowers 2008). Plants on the basis of adaptive evolution can be classified roughly into two major types: the halophytes (that can withstand salinity) and the glycophytes (that cannot withstand salinity and eventually die). Most of major crop species belong to this second category. Thus, salinity is one of the most brutal environmental stresses that hamper crop productivity worldwide (Flowers 2004, Munns and Tester 2008). Initially soil salinity is known to repress plant growth in the form of osmotic stress which is then followed by ion toxicity (James *et al.* 2011). During the initial phases of salinity stress, water absorption capacity of root systems decreases and water loss from leaves is accelerated due to osmotic stress of high salt accumulation in soil and plants, and therefore salinity stress is also considered as hyperosmotic stress (Munns 2005). Osmotic stress in the initial stage of salinity stress causes various physiological changes, such as interruption of membranes, nutrient imbalance, impairing the ability to detoxify reactive oxygen species (ROS), differences in the antioxidant enzymes and decreased photosynthetic activity, and decrease in stomatal aperture (Munns and Tester 2008). Salinity stress is also considered as a hyperionic stress. One of the most detrimental effects of salinity stress is the accumulation of Na^+ and Cl^- ions in tissues of plants exposed to soils with high NaCl

concentrations. Entry of both Na^+ and Cl^- into the cells causes severe ion imbalance and excess uptake might cause significant physiological disorder(s). High Na^+ concentration inhibits uptake of K^+ ions which is an essential element for growth and development that results into lower productivity and may even lead to death (James *et al.* 2011). In response to salinity stress, the production of ROS, such as singlet oxygen, superoxide, hydroxyl radical, and hydrogen peroxide, is enhanced (Apel and Hirt 2004, Mahajan and Tuteja 2005, Ahmad 2010, Ahmad and Prasad 2012, Ahmad and Umar 2011). Salinity induced ROS formation can lead to oxidative damages in various cellular components such as proteins, lipids, and DNA, interrupting vital cellular functions of plants. Grain legumes generally are considered as salt sensitive (Katerji *et al*. 2001). Increase of NaCl concentration reduced the germination percentage, the growth parameters and the relative water content of the lentils. The inhibitory effects of the salinity differed in legume species. The salinity effect on bacterial activity with respect to nitrogen fixation is one of the hypothesis for explaining its salt sensitivity (Pessarakli *et al*. 1989, Katerji *et al.* 2001). Lentil under saline conditions systematically shows lower values of nitrogen fixation during the whole growing season (van Hoorn *et al.* 2001). Under excessively high salinity there is failure of germination or if germination occurs, there is stunted growth with plants developing yellowish discoloration followed by development of bright reddish pigmentation. Nodulation is poor or absent. When salinity develops because of the rise of water table after the crop establishment, the crop stops growing, shows moisture stress by drooping of leaves and after a few days the plants defoliate and die. Selection of salt-tolerant varieties of lentil would best way to cultivate this crop on saline soils. Plants develop various physiological and biochemical mechanisms in order to

survive in soils with high salt concentration. Principle mechanisms include, but are not limited to ion homeostasis and compartmentalization, ion transport and uptake, biosynthesis of osmoprotectants and compatible solutes, activation of antioxidant enzyme and synthesis of antioxidant compounds, synthesis of polyamines, generation of nitric oxide (NO), and hormone nodulation (Elsheikh and Wood 1990).

1.6 Lentil – a nutrient rich crop

A legume belongs to the Fabaceae family. This family is one of the largest families of flowering plants with 18,000 species classified into around 650 genera (Polhill and Raven 1981). The family is usually divided into three sub-families: Papilionoideae, Caesalpinioideae and Mimosoideae. The three subfamilies are generally identifiable by their families.

People have been growing legumes since 6000 years ago. Legumes are grown agriculturally, primarily for their grain seed called pulse, for livestock, forage and as soil-enhancing green manure. Well-known legumes include alfalfa, clover, peas, beans, lentils, soybeans and peanuts. A legume fruit is a simple dry fruit that develops from a simple carpel and usually dehisces on two sides. Legumes are notable in that most of them have symbiotic nitrogen-fixing bacteria in a structure called root nodules. For that reason, they play a key role in crop rotation.

Lentil derived from a Latin word *Lens* that describes exactly the shape of the seed of a cultivated legume, which are called *Lens culinaris* Medik. The scientific name was given

by a German botanist-physician Medikus in 1787. Lentil is under the sub-family Papilionoideae. All *Lens* species are diploid with 2n=14 chromosomes (Sharma *et al.* 1963, Ladizinsky 1979). Lentil is under the sub-family Papilionoideae. Lentil is an important legume because it is a source of high quality protein for human diet and animal consumption (Kurdali *et al.* 1997, Thomson and Siddique 1997, Katerji *et al.* 2001). Lentil contains about twice as much protein as cereals. The seeds of it contain approximately 11.2% water, 25% protein, 10% fat, 55.8% carbohydrates, 3.7% fiber, and 3.3% ash (Purseglove 1968, Wang *et al.* 2009). It becomes very popular among the people of the third world countries as an important protein source. It also contains amino acid lysine which is generally deficient in food grains (Elias *et al*. 1986).

Lentil seeds dating back 8,000 years have been found at archeological sites in the Middle East. Lentil is considered an "ancient crop for modern times" (Sultana and Ghafoor 2008). It is an important pulse which is cultivated throughout the world. In the modern and technologically advanced world, among other crops lentil crop is widely cultivated in more than 35 countries of 5 different continents, throughout Europe, Asia, and North Africa. Pulses are important legume crops worldwide because of their importance in food, feed, and cropping systems. The lentils are grown for their small lens-shaped edible seeds, which are rich in protein (35-40%) and carbohydrates, and are a good source of calcium, phosphorus, iron and B vitamins. Lentil seeds provide high levels of protein and, when consumed in combination with cereals, they provide adequate amounts of essential amino acids for the human diet. Lentil seeds are rich in polyphenols, which have antioxidant, antimutagen and free radical-scavenging abilities and their potential effects

are beneficial to human health in the prevention of many chronic diseases, so the investigation of the polyphenolic content in lentil seeds is worthwhile (Dueńas *et al*. 2002, 2003).

1.7 Ecological requirements of lentil plants

Lentil is a cool season crop and is best grown in soil with pH 6.0-8.0 and cannot tolerate water logging, flooding or high soil salinity. They are generally grown without fertilizer since they can meet their nitrogen requirement by symbiotic fixation of atmospheric nitrogen in the soil (Senanayake *et al*. 1987, Zapata *et al*. 1987, Fride and Middleboe 1977). Salinity is often encountered in irrigated agriculture in areas where the water table is shallow (0.5-3 m) or the irrigation water has a high salt content (>1000 ppm). Lentil is highly sensitive to salinity and suffers from salinity stress. The species is classified as salt sensitive (Ashraf and Waheed 1990) like many other legumes.

1.8 Lentil cultivation in Bangladesh

In Bangladesh, lentil placed second position among the pulses according to area and production but stood first in terms of usage (Hossain *et al* 2018, Afzal *et al.* 1999). It is the principal and popular edible crop among pulses. The area and production of lentil in Bangladesh were 5,15,956 acres and 1,53,000 metric ton in 1991-92 (BBS 1995). After 18 years, the area and production of lentil were 1,75,402 acres and 60,537 metric ton in 2008-09 (BBS 2009). Thus, the area and production of lentil decreased 2.94 and 2.53

times, respectively over 18 year. However, from 2009, the area and production of lentil increased rapidly as shown in Table 1.

Table 1. The area and production of lentil in Bangladesh from 2009-10 to 2015-2016 (BBS 2016).

Year	Area (acres)	Production (metric ton)
2009-2010	190982	71100
2010-2011	205024	80442
2011-2012	213035	80125
2012-2013	222305	93098
2013-2014	307637	157422
2014-2015	359367	167361
2015-2016	381653	158228

Lentil is cultivated in different parts of the country. But, it is extensively cultivated in Jessore (28,003 acres), Faridpur (42,704 acres) and Rajshahi (33,145 acres) in 2016 (BBS 2016) (Fig. 1.2). The yield of local lentil variety in Bangladesh is very poor, but varied widely between farms and between locations (Sikder and Elias 1985). Bangladesh Agricultural Research Institute (BARI) has developed a good number of popular varieties of lentil. At first in 1991, BARI released a lentil variety 'BARI masur-1'. Then, it released BARI masur-2 (1993) and BARI masur-3 (1996). After that, BARI continuously released BARI masur-4, BARI masur-5, BARI masur-6 and BARI masur-7. These are growing in the farmers' fields. However, the adoption status and the economic performance of this crop are unknown to the researchers and policy makers. Because a limited study was done in this line. The rate of adoption and sustainability of any crop depend largely on its economic profitability. Economic viability is one of the important criteria for assessing the suitability of a new crop technology. In addition, challenges

from environmental degradation to the adaptation and growth of lentil crops are also important to take under consideration.

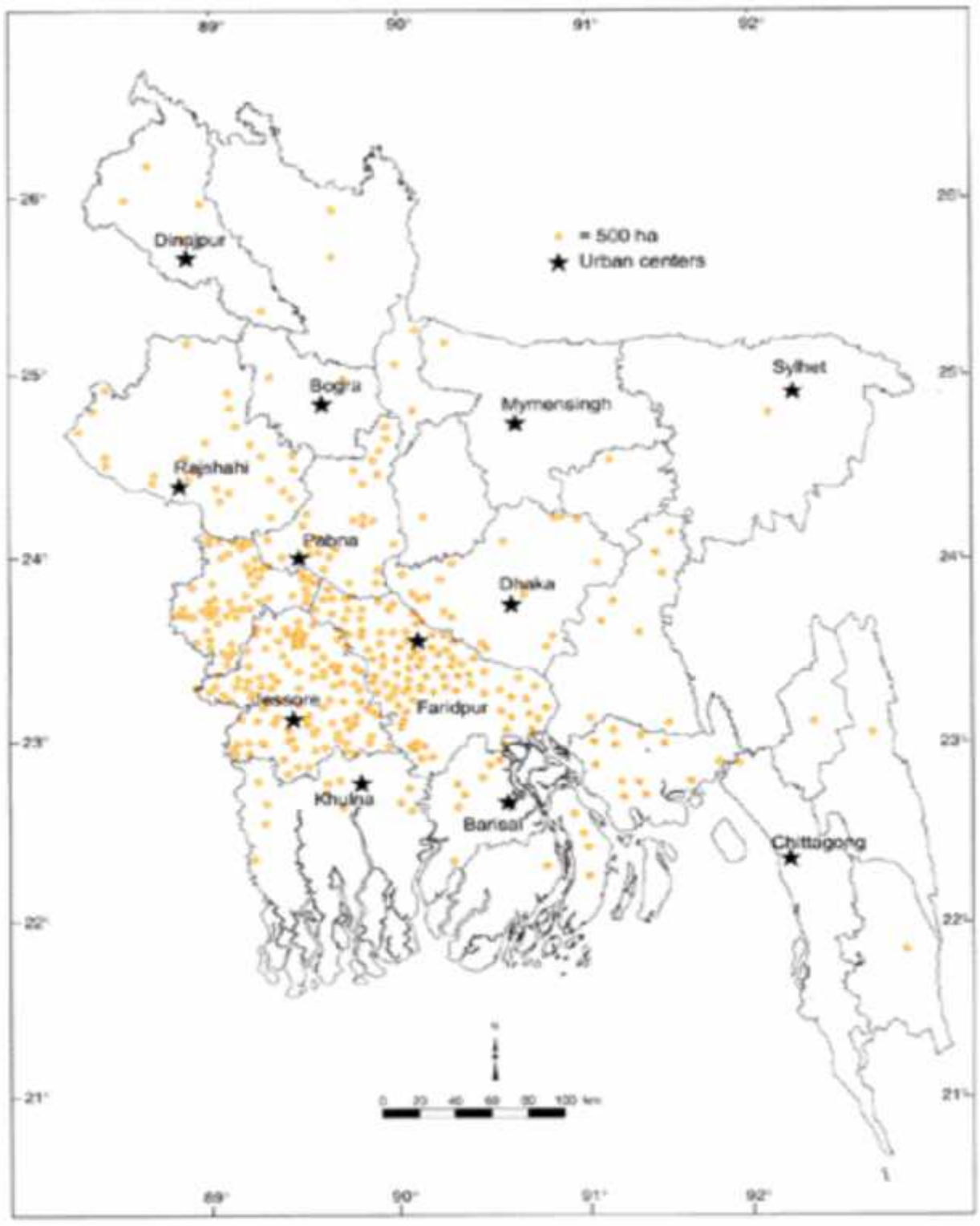

Fig. 1.2. Lentil cultivated areas in Bangladesh.

1.9 Role of legumes in soil nitrogen (N) availability

Growth and development of plants are influenced by a number of physical, chemical and biological factors. Availability of soil mineral nutrients content in turn affects the chemical composition of the plants (Juma and Averbeke 2005). Among available soil mineral nutrients, Nitrogen (N) is one of the essential elements for plant growth. Nitrogen is the most limiting nutrients to crop production (Mugwe *et al.* 2004). Soil organic matter plays an important role in maintaining physical, chemical and biological properties of soil and therefore the plant productivity and crop yield (Micheni *et al.* 2004). Changes in vegetation cover can lead to the changes in soil's physical, chemical and biological properties (Sanchez *et al.* 1997). The lowest N_2 recoveries were associated with a low level of soil organic matter and a high level of N_2 addition. When the soil C/N ratio is small, there free N_2 is assumed to occur (Mac Vicar *et al.*1951). Changes in soil properties and their effects on plants have been reported in plants (Hossain *et al.* 2016b). Leguminous plants are with special features having root nodule compared to plants of other families. It is important to understand the factors that affect the root nodules and the root systems that are related to nodule development and hence nitrogen fixation. Therefore, availability of N_2 in the soil through biological N_2-fixation by legumes play important role in any ecosystem where there exists N limitation. Nitrogen availability is the important limiting factor for plant growth in an ecosystem (Taiz and Zeiger 2006). Biological N_2 fixation in the agricultural systems is important from the aspects of both

economy and environment. Therefore, it is relevant to understand the biological N_2 fixation through symbiotic association between legume and *Rhizobium.*

1.10 Symbiotic association between legume and rhizobia

Symbiotic Nitrogen (N) fixation involves different host plants and various groups of microsymbionts. Organisms that can fix nitrogen, i.e., convert the stable nitrogen gas in the atmosphere into a biologically useful form are generally known as nitrogen fixing organisms. A wide range of organisms have the ability to fix nitrogen. However, only a very small proportion of species are able to do so; about 87 species in 2 genera of archaea, 38 genera of bacteria, and 20 genera of cyanobacteria have been identified as diazotrophs or organisms that can fix nitrogen (Zahran *et al.* 1995). Among the 38 genera of bacteria, Rhizobia are a genetically diverse and physiologically heterogeneous group of bacteria that have their ability to do the nodulation in Fabaceae. Rhizobia include several genera that induce nodule formation in legumes; these are *Rhizobium, Sinorhizobium, Mesorhizobium, Bradyrhizobium* and *Azorhizobium*.

Many leguminous plant species can enter into a symbiotic relationship with these root-nodule forming bacteria rhizobia. Traditionally, three main subfamilies are distinguished under the leguminosae family. These are Caesalpinoidae, Mimosoidae and Papilionoidae. The sub-family Caesalpinoidae has very few nodulating members. On the other hand most of the important agricultural crops belong to the sub-family Papilionoidae. Nitrogen fixing symbiosis has evolved in several lineages, but not all legumes form symbiosis. About 12,000 nodulated legume species are known and each

has its own *Rhizobium* partner(s). Rhizobia are facultative microsymbionts. They are live as normal components of the soil microbial Population. When find specific host legume then they start living symbiotically in the root nodules. Outside the root nodule, rhizobia are mostly found on the rhizoplane soil (i.e. soil around on the root surface) and rhizosphere soil (i.e. soil around the root surface). In non rhizosphere soil, Rhizobia are found to a lesser extent. The excretion of nutrients by plant roots, especially the host legume are mostly available in the rhizosphere soil. For this reason, large numbers of rhizobia are mostly found in the rhizosphere soil. Besides the host legume, nonlegumes, moisture, temperature, acidity alkalinity and salt content of the soil affect rhizobial populations in the soil (Sandhu 2007) Numbers of rhizobia *i*n the soil can range from undetectable to 1,000,000 rhizobia g^{-1} soil. The soil is the major reservoir of free-living rhizobia.

The legume-*Rhizobium* interaction is the result of specific recognition of the host legume by *Rhizobium.* Various signal molecules that are produced by both rhizobia and the legume confer the specificity. Exopolysaccharide (EPS) produced by *Rhizobium* is one such signal for host specificity during the early stage of root hair infection (van Hoorn *et al.* 2001). Hence, only rhizobia are specifically compatible with a particular species of legume. These can stimulate the formation of root nodules which in turn increase nitrogen fixation for better production of legume.

The symbiosis is triggered by nitrogen starvation of the host legume plant. In this situation the host plant has to select its *Rhizobium* partner from billions of bacteria in the

rhizosphere soil (Yang *et al.* 2016). This is achieved by secretion of flavonoid signal molecules from the root of host plant. Flavonoid acts as a chemo-attractants between legumes and rhizobia but most importantly act as inducers of the *Rhizobium* nodulation genes (Senanayake *et al*. 1987). These genes are required for the production of bacterial signal molecules i.e. the Nod factors (NFs). The Nod factors (NFs) trigger the nodule developmental program in the host plant (Singleton and Bohlool 1984).

1.11 Nodule formation by nodulation genes and *nod* factors

Rhizobia are a group of soil bacterial species that infect leguminous plants leading to the formation of special type of organs called nodules, where nitrogen fixation takes place. Different nodulation genes (*nod, nol, noe, nif* and *fix* genes) function as a genetic determinant for rhizobia in the nodule formation. The *nod, nol* and *noe* gene products are involved in production of a nodulation signal. The Nod factor, which is a lipo-chitooligosaccharide which acts as an elicitor of root nodule formation by the plant by triggering a developmental program leading to construction of the root nodule and entry of rhizobia into the nodule (Long 2001, Geurts and Bisseling 2002, Gage 2004). It is an important host specificity determinant (Spaink 2000). The host plants also produce flavonoids in the rhizosphere. These signals act as inducers of a specific bacterial nodulation genes. Initiation of nodule formation on compatible host plants results from a molecular dialogue between the host and the bacteria (Dénarié *et al.* 1993, Schultze and Kondorosi 1998, Perret *et al*. 2000, Spaink 2000). The nitrogen fixing genes are located in rhizobia and symbiotic establishment and regulation of nitrogenase activity in the nodules depend on the genes of host plant.

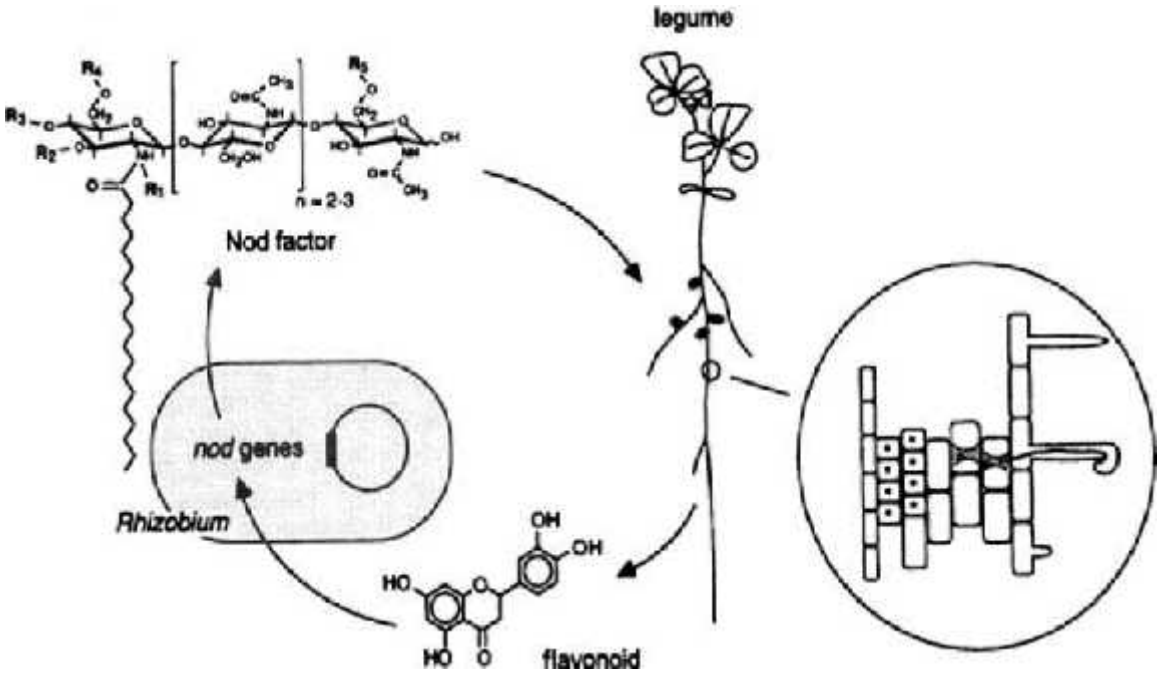

Fig. 1.3. Process of root nodule formation in legume plants.

At the beginning of the nodule formation, the free-living rhizobia in the soil enter the root hairs of the susceptible host legume by a complex series of interactions which is known as the infection process. This begins with the adhesion of the specific rhizobia to the surface of the root hair and curling the root hair. The hypha-like infection thread develops gradually in the root hair. The infection thread contains large numbers of rhizobial cells. In the infection area of the root hair cells gradually forms into a tubular like structure which is known as root nodules (Fig.1.3). Generally branched rods (X- and Y-shaped) and large pear-shaped rounded form nodules are found in the legume. The rhizobia are released from the tip of the infection thread into the cytoplasm of the host cells. Before the release of the rhizobia, rapid host cell division takes place. The

host cell membrane, which had enclosed the infection thread, buds off vesicles containing the rhizobia.

1.12 Taxonomy of *Rhizobium*

The bacteria which form nitrogen-fixing symbiosis with legume plants belonging to diverse groups of α- and β-proteobacteria are collectively called Rhizobia (Chen *et al.* 2003, MacLean *et al.* 2007). About 88% of the legume species are found to form nodules in association with a group of bacteria called rhizobia and fix nitrogen (Graham and Vance 2003). These rhizobial bacteria belong to the large Proteobacterial Phylum Rhizobia belonging the family Rhizobiaceae that consists of the Genera: alpha-proteobacteria and beta-proteobacteria. The alpha-proteobacteria genera consist of the following species: *Agrobacterium, Allorhizobium, Rhizobium, Bradyrhizobium, Sinorhizobium, Mesorhizobium, Azorhizobium, Devosia, Methylobacterium, Ochrobactrum and Phyllobacterium* (Freidberg *et al.* 1997). They are all nodule-forming bacteria. Among them, *Rhizobium* spp. are fast growing and *Bradyrhizobium* spp. are the slow growing bacteria. On the other hand, the beta-proteobacterial genera consist of the following species: *Burkholderia* and *Cupriavidus* (Lindstrom and Martine-Romeo 2007). These all are Gram-negative bacteria. However, classification of rhizobia is becoming increasingly complex and is revised periodically because of new findings that propose new genera and new species. Rhizobia are somewhat unique among all soil bacteria because they are able to infect the roots of a leguminous plant and leads to the formation of root nodules where the conversion of atmospheric N_2 to ammonia occurs. Legume roots are infected by specific groups of rhizobia. In agriculture, use of compatible and

efficient *Rhizobium* strain determines optimum crop productivity in terms of plant biomass and total amount of fixed N in plant tissue. The systematic position of *Rhizobium* is as follows:

Kingdom: Bacteria

Phylum: Proteobacteria

Class: Alphaproteobacteria

Order: Rhizobiales

Family: Rhizobiaceae

Genus: *Rhizobium*

1.13 Characteristics of rhizobial bacteria

Rhizobia are the soil bacteria having the ability to induce nitrogen-fixing nodules on roots in lentil as well as other legume crops. Rhizobia are mainly medium sized and rod-shaped cells. Their width is 0.5-0.9µm and length is 1.2-3.0µm. They do not form endospores (Compant *et al.* 2010). They always form exospores. They are Gram-negative in nature. They are mobile by a single polar flagellum or two to six peritrichous flagella. Rhizobia are predominantly aerobic and chemo organotrophs. They are relatively easy to be cultured. They grow well in the presence of O_2, and utilize relatively simple carbohydrates and amino compounds. Some strains of rhizobia require vitamins for growth. Optimal growth of most strains occurs at a temperature range of 25-30^{0}Cand a pH of 6.0-7.0. However, *Rhizobium* spp. are likely to lose viability rapidly in water.

Generally, most rhizobia produce white colonies. However, the first growing *Rhizobium* spp. generally produce pinkish colony in YEM medium. Most rhizobia only weakly absorb Congored (diphenyldiazo-bis-α-naphthylaminsulfonate) dye, which is included in culture media for isolating rhizobia. Other interesting and useful characteristics of rhizobia are their growth reaction in the standard YEM medium containing bromothymol blue as the pH indicator. Fast growing rhizobia produce an acid reaction in the YEM medium containing bromothymol blue (pH 6.8). On the other hand, slow growing rhizobia produce an alkaline reaction in the YEM medium containing bromothymol blue. With the exception of a few strains, rhizobia have not been found to fix N_2 in the free-living form except under special condition. They are able to fix N_2 by formation of symbiotic association with legumes (Zahran 1999).

1.14 Significance of biological nitrogen fixation for soil fertility

Rhizobia are somewhat unique among soil microorganisms for their ability of biological N fixation through symbiotic association with legumes. The enzyme which is called nitrogenase is responsible for the conversion (reduction) of atmospheric N_2 into NH_4. The legumes utilize this NH_4 and convert it into amino acids, which in turn are synthesized into proteins. The complex biochemical process by which the atmospheric nitrogen is enzymatically reduced into utilizable form for the plant by the nitrogenase enzyme complex of the rhizobia is called biological nitrogen fixation.

Biological nitrogen fixation is a phenomenon occurring in all known ecosystems and is undoubtedly of greatest agricultural importance. Atmospheric N fixed symbiotically by

the association between *Rhizobium* species and legumes represents a renewable source of N for agriculture (Peoples *et al*. 1995b). Biological nitrogen fixation is an efficient source of nitrogen (Peoples *et al*. 1995a). The total annual terrestrial inputs of N from biological nitrogen fixation ranges from 139 million to 175 million tons of N, with symbiotic associations growing in arable land accounting for 25 to 30% (35 million to 44 million tons of N) and permanent pasture accounting for another 30% (45 million tons of N) (Burns and Hardy 1975). Above these figures illustrated the relative importance of biological nitrogen fixation in cropping and pasture systems. Values estimated for various legume crops and pasture species are often impressive, commonly falling in the range of 200 to 300 kg of N ha^{-1} $year^{-1}$ (Peoples *et al*. 1995a). Yield increases of crops planted after harvesting of legumes are often equivalent to those expected from application of 30 to 80 kg of N fertilizer ha^{-1}. It also shows that there is a proportion of the 80 to 90 million tons of N fertilizer expected to be applied annually in the agricultural land by the end of the decade (Peoples *et al.* 1995a). If nitrogen is fixed biologically through the symbiotic association than this proportion of N fertilizer application should be reduced which is very beneficial for our environment. Much of the land has been degraded worldwide due to the various reasons. Now it is time to stop the destructive uses of land and biological nitrogen fixation can also play a key role in land remediation.

The symbiotic associations between leguminous plants and rhizobia have the greatest quantitative impact on the nitrogen cycle. A tremendous potential for contribution of fixed nitrogen to soil ecosystems exists among the legumes (Brockwell *et al.* 1995, Peoples *et al.* 1995, Tate 1995). There are approximately 700 genera and about

13,000 species of legumes, only a portion of which (about 20%) have been examined for nodulation and shown to have the ability to fix N. Estimates are that there are more than 100 agriculturally important legumes which contribute nearly half the annual quantity of biological nitrogen fixation entering soil ecosystems through rhizobial symbioses (Tate 1995).*Rhizobium* is also a plant growth promoting rhizobacteria (PGPR) which exhibit a variety of characteristics responsible for influencing plant growth and performance. As a symbiotic partner, in addition to nitrogen supply, *Rhizobium* also improves nutritional uptake by promoting the growth of plant root system through production of Indole acetic acid.

Legumes are very important both ecologically and agriculturally because they are capable to fix atmospheric N in various forms such as ammonia, nitrate, organic nitrogen and maintain substantial part of the global flux of nitrogen. Whatever the true figure, legume symbioses contribute at least 70 million tons of N per year, approximately half deriving from the cool and warm temperature zones and the remainder deriving from the tropics (Brockwell *et al*. 1995). Increased plant protein levels and reduced depletion of soil N reserves are obvious consequences of legume N fixation. Deficiency in mineral nitrogen often limits plant growth. However in this case, symbiotic relationships have evolved between plants and a variety of nitrogen-fixing organisms (Freiberg *et al*. 1997).

Most of the attention is directed toward N fixation by legumes because of their ability to fix N and their contribution to integral agricultural production systems in both tropical and temperate climates (Peoples *et al*. 1995b). Successful *Rhizobium*-legume symbioses

will definitely increase the quantity of biological nitrogen fixation into soil ecosystems. *Rhizobium*-legume symbioses are the primary source of fixed nitrogen in land-based systems and can provide all over half of the biological source of fixed nitrogen (Tate 1995).

However, the measured amounts of N fixed by symbiotic systems may differ according to the method used to study N fixation (Sellstedt *et al.* 1993). In a terrestrial ecosystems, the inputs of total biological nitrogen fixation from the symbiotic relationship between legumes and their rhizobia amount to at least 70 million tons of N_2 per year (Brockwell *et al*. 1995). This enormous quantity will have to be decreased because of increasing the world's population and diminishing the natural resources that supply N fertilizer. However this objective will be achieved through the development of superior legume varieties, improvements in agronomic practice, and increased efficiency of the nitrogen-fixing process itself by better management of the symbiotic relationship between plants and rhizobia. However, the symbioses between *Rhizobium* and legumes are a cheaper and usually more effective agronomic practice for ensuring an adequate supply of N for legume-based crop and pasture production than the application of N fertilizer.

1.15 Effects of salinity on biological nitrogen fixation

Biological nitrogen fixation is an efficient source of nitrogen (Peoples *et al.* 1995a). Biological N fixation by the symbiotic association with rhizobia are influenced by a number of environmental factors such as soil pH, temperature, drought, salinity, flooding, competition, plant stress, N level, seed treatments, chemicals or pesticides, additional concerns and organic matters in the soil (Peoples *et al.* 1995a). Among the different

environmental factors, salinity is a major stress factor that suppresses the growth and symbiotic characteristics of most rhizobia. Salinity stress significantly limits productivity of legume because of its adverse effects on the growth of the host plant and root nodule bacteria, symbiotic development and N fixation efficiency (Rai 1992). The formation of nodule, therefore, is decreased significantly due to the salinity. Soil salinity adversely affects the nodulation and nitrogen fixation capacities of rhizobia, resulting in lower productivity of legumes. Salt stress not only inhibits the initial steps of nodule initiation, nodule infection and development but also has a depressive effect on nitrogen fixation (Zahran 1999). These symbiotic association may be influenced by a number of other physical factors of which availability of N_2 fixing bacteria is one of them (Date 1970). The efficiency of biological nitrogen fixation mainly depends on the host cultivar and rhizobia.

Salinity affects the infection process by inhibiting root hair growth. Legumes grown in saline environment exhibit reduced number and weight of root nodules. The amount of nitrogen fixed per unit weight of nodules is also decreased due to the salinity (Serraj 2002). Thus, in saline soils the yield of leguminous crops is decreased due to the lack of successful symbiosis (Hafeez *et al*.1988). Excess salt in the soil adversely affects survival, growth, nodulation, nitrogen fixation and legume-*Rhizobium* symbiosis (Rai 1999). Unsuccessful symbiosis under salt-stress may be due to failure in the infection process due to the salinity on the establishment of rhizobia. Nitrogenase activity was substantially inhibited by sodium chloride. This inhibition was associated with a significant decrease in nodule permeability to oxygen diffusion in several legume species

as reported by Serraj (2002). If salt-tolerant lentil genotypes and their *Rhizobium* strains are identified then there is a potential to enhance N fixation by using salinity tolerant rhizobial strain compatible to legume crops under saline zone. Salt tolerance of symbiotic nitrogen fixation reportedly depends both on the plant and the *rhizobium* genotype. There is a great possibility to increase its production by exploiting better colonization of their root and rhizosphere through *Rhizobium* bacteria, which can reduce nitrogenous fertilizer use and can protect environment.

1.16 Molecular markers used to study genetic diversity of *Rhizobium*

Molecular markers are used to identify a particular sequence of DNA. Molecular marker also known as genetic marker is a fragment of DNA sequence. This is associated with the part of the genetic material. As the DNA sequences are very highly specific, so that they can be identified with the help of the known molecular markers. Molecular markers can be used to find out a particular sequence of DNA from a group of unknown DNA sequences.

Molecular markers are widely used to understand the polygenic traits based on genetic material. Various molecular techniques are used to identify various soil bacteria**.** Among various molecular techniques, whole cell protein analysis, detection of specific DNA sequences by hybridization restriction digestion and sequence comparison have been used for assessing the diversity among soil bacteria. The rRNA genes are allied component of DNA of all organisms and 16S rRNA is specifically highly conserved in bacteria. These 16S rRNA is being used as biological marker for identification of species, genus and

families. There are two kinds of regions those are known as conservation area and variable area (Fig.1.4).

Using different methods, sample variations can be detected as well as the quality of information content (Sultana and Ghafoor 2008). In many crops, protein electrophoretic markers have been used in some cases. However. the major limitation of this marker is the lack of enough polymorphism among closely related cultivars. For this reason, DNA-based genetic markers have been recently integrated into several plant systems and are playing a very important role in molecular genetics and plant breeding (Beckmann and Soller 1993). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) of seed proteins is a practical biochemical technique and reliable method to detect the biochemical markers of lentil especially for the cultivar levels due to the independence of environmental fluctuation (Murphy *et al*. 1990, Yuzbasßioglu *et al.* 2008). Also SDS–PAGE of seed storage proteins provides a powerful, relatively convenient and rapid method of identification and classification of gene bank collection (Cooke 1984, Przbylska 1986, Pedalino *et al*. 1992), that is used to distinguish between genotypes (Ismet and Fikret 2011). Therefore, the choice of the technique depends on the objective of the study, sensitivity level of the marker system, financial constraints, skills and facilities available (Yoseph *et al*. 2005). Among the different molecular marker approaches employed in fingerprinting, the inter simple sequence repeat (ISSR) technique is a quick, reliable and highly informative technique (Reddy *etal.* 2002). Inter simple sequence repeat (ISSR) marker, in addition to its suitability to genetic diversity study, is highly polymorphic, reproducible, cost effective, and requires no prior

information of the sequence (Bornet *et al.* 2002). These facts suggest that ISSR could be an unbiased tool to evaluate the changes of diversity in agronomically important crops (Brantestam *et al.* 2004).

Various molecular techniques have made easy the way to indentify and classify nitrogen fixing bacteria. These nitrogen fixing bacteria can be identified and classified on the basis of DNA sequences obtained by using various molecular markers. There have been many advanced and powerful tools used in molecular biology which are mainly PCR-based. Different plant genotype has different fragment profile which can be used as a genetic marker. This genetic marker can be used for genotypic identification, genome mapping and gene tagging. During the past decade ribosomal RNA (rRNA) based identification and detection methods have become a major technique that used in all disciplines of microbiology. Although, 16S rRNA gene is used commonly to identify various genera and species, due to its conserved-sequence nature. The 16S rRNA gene is useful for characterization because it is slowly evolving.

Differentiation between *Rhizobium* species can be estimated by comparison of 16S rRNA gene sequences amplified by polymerase chain reaction. Restriction fragment polymorphism (RFLP) analysis of 16S rRNA sequences amplified by polymerase chain reaction has permitted to estimate the variation in 16S rRNA. Sometimes PCR based genomic finger printing methods provide much more fine taxonomic resolution than 16S r-RNA sequencing (Freiberg *et al.* 1997).

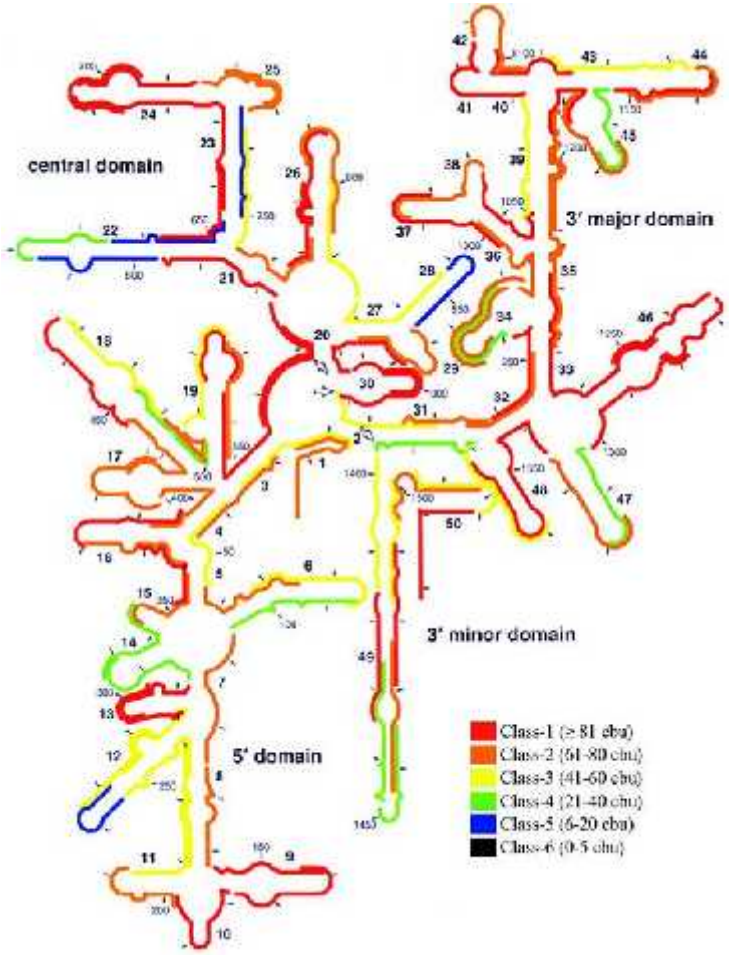

Fig. 1.4. Structure of 16s ribosomal RNA short subunit.

1.17 Objectives of the research work

The main objective of the research was to study the effects of salinity on the nodulation and growth of *Lens culinaris* Medik. as well as the genetic diversity of rhizobia compatible to lentil plants.

The specific objectives of the research were to study

1. the effect of salinity on the growth parameters of lentil plants
2. the effect of salinity on the nodulation (number, size and efficiency) of lentil crops and
3. the identification and diversity of nodule-forming rhizobia compatible to lentil by using molecular marker 16s rRNA gene.

2. MATERIALS AND METHODS

2.1 Effects of salinity on the growth and development of lentil plants

2.1.1 Seed selection and surface sterilization

Seeds of BARI masur-4 was collected from Bangladesh Agricultural Research Institute (BARI), Joydevpur, Gazipur. Lentil seeds were taken into 50 ml falcon tube and seeds were surface-sterilized with 5% sodium hypochlorite (NaOCl) (Sauer and Burroughs 1986). Tubes were shaken gently for 10 minutes at about 400 rpm. Then, the seeds were washed by rinsing with autoclaved distilled water at least 5 times.

2.1.2 Salt treatment

The plants treated with NaCl were divided into four groups based on the molarity of NaCl: Control (NaCl free), 50 mM NaCl, 100 mM NaCl and 200 mM NaCl. To prepare 50 mM NaCl, 2.93 g NaCl was added into one litre of water. Similarly, to prepare 100 mM NaCl, 5.85 g NaCl was added into one litre of water and for 200 mM NaCl, 11.7 g NaCl was added into one litre of water. After germination of the seeds, salt solution was applied after every 7 days.

2.1.3 Experimental plot preparation

For each treatment, three plots were prepared at the Botanical Garden of University of Dhaka. Thus a total of 12 plots, each 3 for each of the 4 treatments, were prepared. The size of each plot was 0.0625 m^2.

2.1.4 Growing plants

After surface sterilization, seeds were kept in a Petri-dish for germination. Germinated seeds (2 days old) were sown in the plots. Four treatments of 0 mM, 50 mM, 100 mM and 200 mM NaCl were applied. Each time 150 ml salt solutions were applied after every 7 days in each plot of all treatments. Plants were harvested from the field after 2 months of sowing. Average height (in centimeters) of 5 plants per plot was recorded randomly.


a
b
c

Fig. 2.1. Experimental field at the Botanical Garden at Curzon Hall area, University of Dhaka. After 21 days (a and c) and after 14 days (b).

2.1.5 Determination of shoot height

A centimeter scale was used to measure the height of plant shoots. Height was recorded from the surface of soil where shoot starts. The mean value of height of 5 plants was taken for the determination of height for each plot. Shoot height was recorded after harvesting the plant.

2.1.6 Measurement of root length

After separating from the roots, roots were washed with water. Root systems were spread on a tray with water to measure the length of root systems. A scale was placed on the tray in such a way that roots were which was parallel to the length of the root system and then the length was noted.

2.1.7 Determination of shoot fresh and dry biomass

After harvesting, plant shoots were cut with the help of a scissor. Then, plant shoots were weighed for the fresh biomass. Shoots were kept into a separate envelop and then oven dried at 80^{o}C for 24 hours. Dry shoots were weighed for the dry biomass. Biomass was expressed as gram per plant.

2.1.8 Determination of root fresh and dry biomass

After separating shoots from root, the root systems were cleaned carefully by gentle flow of tap water so that roots are not lost. Excess water was removed from the root with the help of tissue paper. Then, fresh weight of root was taken. Roots were kept into a separate envelop and then oven dried at 80ºC for 24 hours. Dry roots were weighed for the dry biomass and expressed as gram per plant.

2.1.9 Determination of leaf chlorophyll content

Leaf chlorophyll content was determined with the help of a chlorophyll meter (SPAD-502Plus, Konica, Minolta, Japan). In order to determine chlorophyll contents of leaves, 4 fully expanded youngest leaves were selected per plant. The machine was calibrated. Then, mean SPAD value was recorded after harvesting the plants. SPAD values were determined carefully for lentil plant because the leaves of lentil plant were too small to cover the sensor area of the chlorophyll meter.

2.1.10 Extraction and determination of proline

At first 1g of fresh leaf was taken into a clean morter. The leaf was homogenized with 5 ml 0.1 M sulphosalisylic acid. After centrifuging, supernatant was taken and volume of supernatant was adjusted to 5 ml distilled water. Then, 5 ml glacial acetic acid and 5 ml acid ninhydrine was added to 2 ml of supernatant in test tube and shaken vigorously. Mixture was boiled for 1 hour in a water bath. After cooling the tubes, the mixture was extracted with 10 ml toluene in separating funnel. The upper dark red layer was collected and allowed to stand for sometimes. The optical density (OD) was recorded at

520 nm wavelength where toluene was used as blank and proline content was expressed as μg/cm^2.

Calculation of proline content:

Amount of proline in total amount of extract = Concentration from graph ×Molecular weight of Proline (0.115) × final volume.

$$\text{Amount of proline per gram leaf} = \frac{\text{Amount of proline in total amount of extract}}{\text{Fresh weight of leaf}}$$

2.1.11 Collection of root nodule

Fresh and healthy root nodules were collected from each plant. The nodules were usually light brown or pinkish in color, which indicates that active nitrogen fixation has been established between the nodule bacteria and the lentil plant.

2.1.13 Data analysis

ANOVA was done for the tests of significance plant growth parameters by using JMP 4.0 software (SAS Institute, Carry, NC, USA). Tukey HSD tests were performed to compare the means among the treatments.

2.2 Effects of salinity on the bacterial population

2.2.1 Collection of root nodule

Lentil plants were collected for root nodules from different parts of Bangladesh like saline zone of Patuakhali, and non-saline zones such as Cumilla and Rajshahi districts. Samples were also collected from lentil plants grown in the plot of the Botanical garden at Curzon hall area in the University of Dhaka where different salt concentrations (0 mM NaCl, 50 mM NaCl,100 mM NaCl and 200 mM NaCl) were applied. Fresh and healthy root nodules were collected from each plant for the present study. The selected nodules were usually light brown or pinkish in color, which indicates that an active nitrogen symbiosis was established between the nodule bacteria and the lentil plant.

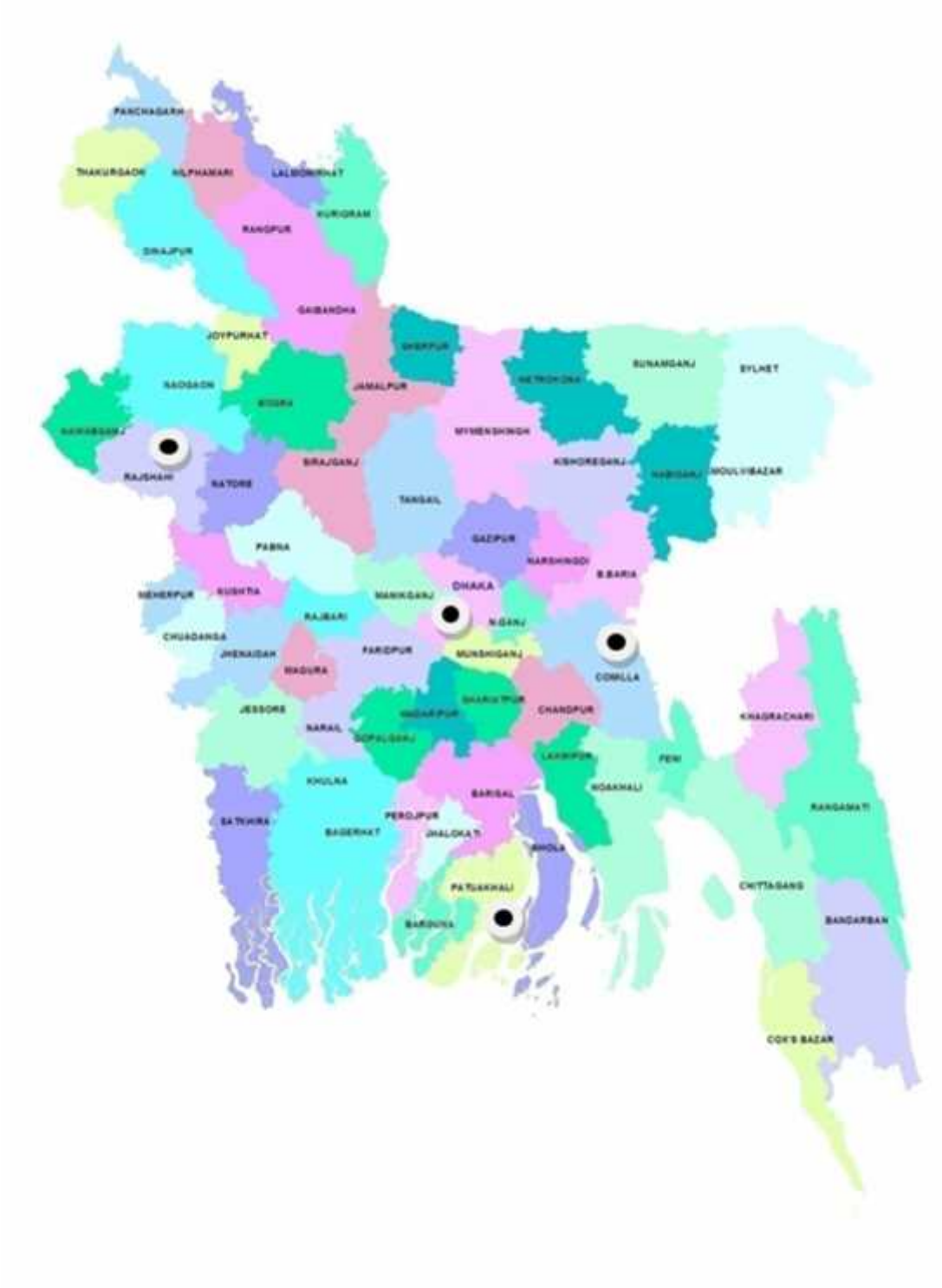

Fig. 2.2. Sampling sites (round symbol) selected for the collection of lentil plants used in the present study.

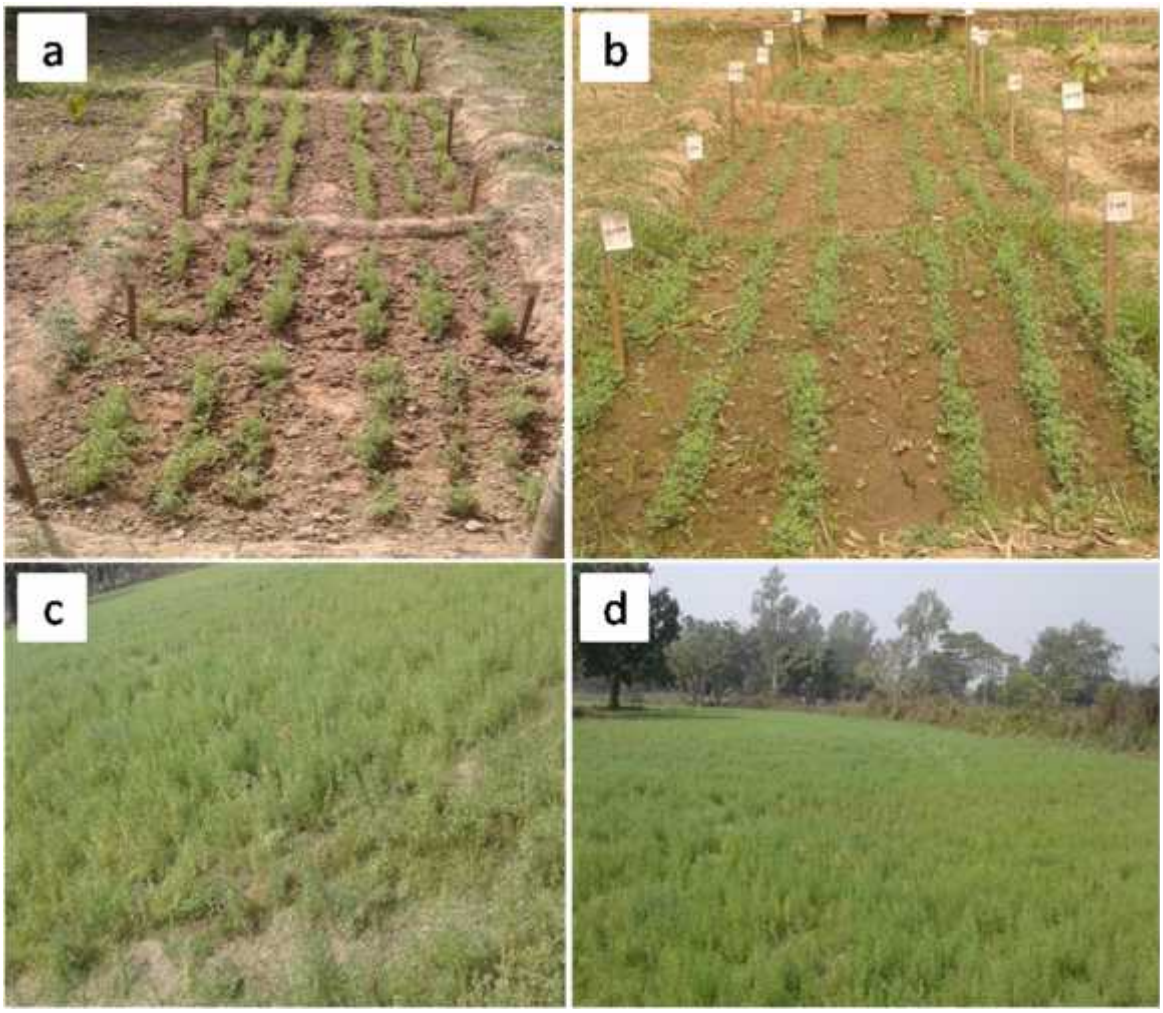

Fig. 2.3. Sites selected for the collection of root nodule: Botanical Garden at Curzon Hall area, Univesity of Dhaka (a); lentil field in Cumilla (b); lentil field in Rajshahi (c).

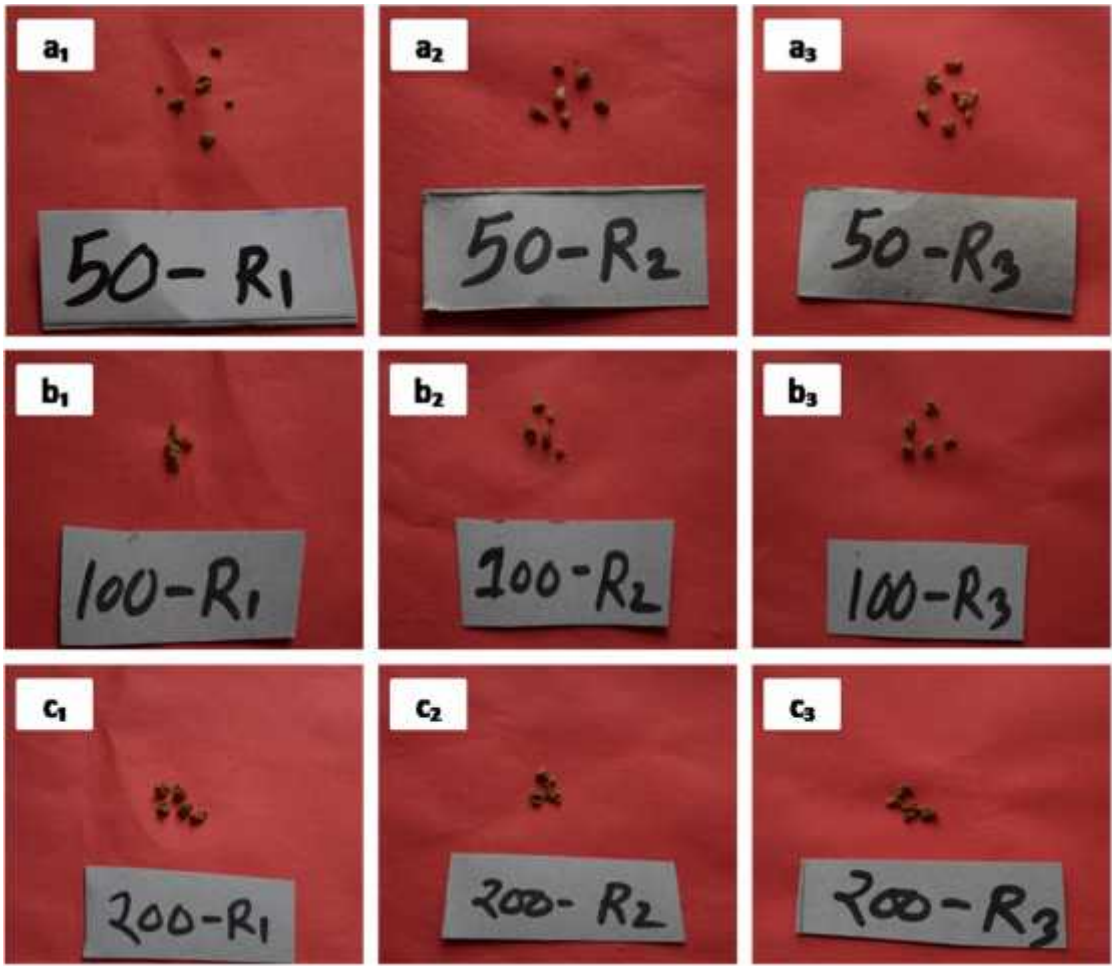

Fig. 2.4. Root nodules collected from the lentil plant grown under different concentrations of salt treatment: 50 mM NaCl (a_1, a_2 and a_3); 100 mM NaCl (b_1, b_2 and b_3); 200 mM NaCl (c_1, c_2 and c_3).

2.2.2 Collection of rhizosphere soil and bulk soil from the field

Comparison of bacterial population density was studied by using samples collected from the plants grown in the plots established in the Botanical Garden at the Curzon Hall area, University of Dhaka. Besides collection of root nodules, rhizosphere soil and the bulk soil samples were also collected from the field for study bacterial population density. To obtain rhizosphere soil, plants with adhering soil were removed from the field and the roots were shaken to remove loose soil for collection of soil samples. Soil aggregates, larger than 1 cm in diameter remaining on roots, were gently crushed with sterile forceps to remove soil not adhering to the root. Soil that remained attached to the roots after this procedure was considered as rhizosphere soil (Angle *et al.* 1996). Then, soil not adhered with the roots in the field where treatments were applied was collected as bulk soil.

2.2.3 Surface sterilization of the root nodules

Healthy and unbroken nodules of lentils were used for the isolation of root nodule bacteria. Nodules were separated from roots with the help of sterilized forcep then washed thoroughly with the flow of sterilized water. After that, nodules were taken into a falcon tube. Nodules were soaked with 2-3% sodium hypochlorite for 10 minutes. Then, sodium hypochlorite was discarded and nodules were rinsed five times with autoclaved water. Nodules were soaked into 70% ethanol for 1 minute. Then, 70% ethanol was discarded and washed repeatedly five times with autoclaved water.

2.2.4 Isolation of bacterial cells from nodule

After surface sterilization, nodules were taken into a watch glass. Then, 200µl of distilled water was added and then crushed with the help of a sterilized metallic rod. The suspension was homogenized. The suspension was taken into a autoclaved falcon tube and kept for few minutes. After about 15 minutes, the upper thin suspension was poured into another autoclaved eppendorf tube which contained the bacterial cells.

2.2.5 Preserving the root nodules

The collected fresh nodules were stored in the refrigerator. A screw cap of glass vial and cotton wool were required. In a glass vial kept the desiccant at the bottom then kept cotton wool were used along with $CaCl_2$ for the preservation of root nodules.

2.2.6 Preparation of Congo red-YMA medium (for 500 ml)

In the present study, Congo red-YMA medium was used for the culture of bacteria inhabiting nodule and also rhi**z**osphere soil and the bulk soil. The following ingredients were required for medium preparation:

Ingredients	**Amount**
Mannitol	5.0 g
K_2HPO_4	0.25 g
$MgSO_4.7H_2O$	0.1 g
NaCl	0.05 g
Yeast extract	0.2 g
Agar	7.5 g
Congo red	0.5 g
Water	upto 500 ml

2.2.7.1 Procedure for preparation of the medium

All required chemicals, except agar and Congo red with appropriate weight, were measured and then was put into a conical flask. Then, sterilized water was added to make whole volume up to 500 ml. After that it was mixed very well by using a glass rod so that all the chemicals are dissolved. Then, agar was added and it was dissolved. Congo red was added accordingly. The medium was autoclaved at 121^0C for 10 minutes. After autoclaving is complete, about 20 ml the medium was poured into each Petridish.

2.2.7.2 Requirements for plating

The following materials were required for platting the bacterial cell:

1. 70% alcohol
2. Spirit
3. Spreader
4. Match
5. Marker/ Lighter
6. Simple scotch tape
7. Medium plate
8. Garbage container
9. Cotton
10. Sample
11. Tips
12. Micropipette
13. Laminar Air flow

2.2.7.3 Plating of the bacterial suspension in the medium

After preparing the medium plate, 20 µl of the bacterial suspension was spread on to the Congo red-YMA media plates. Then, the plates were incubated for overnight at 37 °C to grow and form colonies. After 24h of incubation, the number of colonies per plate was counted. After counting the number of colonies, the plates were preserved at 4 °C for reuse.

2.2.7.4 Maintenance of the cultures

The isolates were sub-cultured (2^{nd} plating) on YMA media, growth was observed in incubator at 37^0C. For long time storage, liquid YMA media were used. One loop was selected from one colony and then it was plunged in the medium. Media were kept in shaker for bacterial growth at room temperature.

2.2.8 Morphological colony characteristics of the isolates

Colonies were checked for their color, shape and size. Most of the colonies were pink in color and rest of the colonies were white. Different sizes of colonies were observed and named by small (<1 mm), medium (1-3mm) and large (>3 mm).

2.2.9 Data analysis

Two-way ANOVA statistics were done for the elucidation of the effects of rhizosphere soil and bulk soil on different sizes of bacterial colonies (small colony, medium colony and large colony) after 24 hours by using JMP 4.0 software (SAS Institute, Carry, NC, USA).

2.3 Identity and genetic diversity of rhizobia

2.3.1 Requirements for bacterial DNA isolation

The following equipments and chemicals were used for bacterial DNA isolation.

a. Bacterial colony

b. Autoclave machine

c. Beaker

d. Micro centrifuge machine

e. Conical flasks

f. Eppendorf tubes

g. Gloves

h. Ice box

i. Water bath

j. Micropipettes and nuclease free micropipette tips

k. pH meter

l. Refrigerator

m. Water distillation plant

n. Fume hood

2.3.2 Extraction of DNA from bacterial colony

Colonies of bacteria were taken in an eppendorf tube and 100 µl distilled water was added with it. Then, it was kept in a water bath for boiling at 100^0C for 10 minutes. Then it was cooled in ice. It was centrifuged at 13,000 rpm for 15 minutes. After centrifugation at 13,000 rpm, DNA present in the supernatant was stored at -20º C for further analyses.

2.3.3 Estimation of quality and quantity of isolated DNA samples

Before PCR amplification, the quality and quantity of isolated DNA samples were measured. Measurements of isolated DNA concentrations were done by estimating the absorbance of DNA by spectrophotometer at 260 nm and 280 nm. Then, the 260:280nm ratio was calculated. The ratio felling between 1.7 and 1.9 indicates the better quality and quantity of isolated DNA samples.

2.3.4 Requirements for PCR

The following chemicals were used for Polymerase Chain Reaction (PCR)

1. Sterile de-ionized distilled water
2. *One Taq* 2X Master mix with standard Buffer
3. Primer (F 16s rRNA)
4. Primer (R 16s rRNA)
5. Template DNA 50 ng/µl

2.3.5 Primers used

In the present study, the following primers were used to identify nodule forming bacteria in the root nodule of lentil.

1. 5′-16s rRNA	CCA GAC TCC TAC GGG AGG CAG C
2. 3′-16s rRNA	CTT GTG CGG GCC CCC GTC AAT TC

2.3.6 Preparation of working concentrations of DNA samples for PCR reaction

The original stock concentration of each DNA sample was adjusted to a unique concentration, 50 ng/µl using the following formula:

$$S_1 \times V_1 = S_2 \times V_2$$

$$V_1 = S_2 \times V_2 / S_1$$

Where, S_1= Stock DNA concentration (ng/µl), V_1= Required volume (µl), S_2= Working DNA concentration (ng/µl), V_2= Working volume of DNA solution (µl). Original stock DNA (2 µl) was taken in a 2 ml eppendorf tube and required amount of TE buffer calculated from the above formula was added to it. Required volume of TE buffer for each sample was calculated and shown.

2.3.7 Preparation of primer

A 100 µM stock solution was prepared as per following procedure:

1. The number of nanomoles (nm) provided (information found on the tube label and/or the technical datasheet) was taken and multiplied the number of nanomoles by ten.

2. The result provided the number of microliters of liquid (TE, Tris, water or another buffer as required) to be added to the tube for reaching a final concentration of 100 µM.

3. The calculated microliters of TE were added to each tube following the respective value and 100 µM concentrated primer stock was prepared and diluted 50 times to make 2µM working solution. This was equivalent to a stock solution of 100 pmol/µl. The stock solution was then further diluted as necessary based upon the application requirements. The stock solution was stored at -20 °C in a non-frost-free freezer, avoiding multiple freeze-thaw cycles.

2.3.8 Preparation of PCR reaction mixture

The following components were used to prepare PCR reaction mixture. The total volume of PCR reaction mixture was 375 µl for 22 samples.

Components of PCR reaction mixture (for 22 reactions):

Sl. No.	Reagents	Amount per sample	Total
1	Sterile de-ionized distilled water	10.5 µl	231 µl
2	*One Taq* 2X Master mix with standard Buffer	12.5 µl	275 µl
3	Primer (F 16s rRNA)	0.5 µl	11 µl
4	Primer (R 16s rRNA)	0.5 µl	11 µl
5	Template DNA 50 ng/µl	1.0 µl	22 µl
	Total	25.0 µl	550 µl

During the experiment, *One Taq* 2X Master mix with standard buffer, primers and DNA sample solution were thawed from frozen stocks, mixed well by vortexing and kept on ice.

2.3.9 PCR conditions

PCR amplification was done in an oil-free thermal cycler (Biometra, UNO II). The optimum amplification cycle was as follows:

	Initial denaturation	94 ºC	For	30 seconds
30 Cycles	Final denaturation at	94 ºC	for	30 seconds
	Annealing at	65 ºC	for	30 seconds
	Extention at	68 ºC	for	1 minute
	Final extension at	68ºC	for	5 minutes

After completion of cycling program, the reactions were held at 4º C.

2.3.10 Polymerase Chain Reaction (PCR)

PCR was performed by following the conditions and steps described above.

2.3.11 Requirements for Agarose gel electrophoresis

The following equipments and chemicals were used to conduct Agarose gel electrophoresis:

a) A horizontal electrophoresis chamber and power supply

b) Gel casting tray and combs

c) Gel Documentation System

d) Gloves

e) Pipette and tips

f) 1.0 kb DNA ladder

g) Electrophoresis buffer (TAE)

h) 6ò sample loading buffer

i) Agarose

j) DNA stain (Ethidium bromide)

2.3.12 Preparation of stock solutions used for Gel–electrophoresis

For conducting the gel electrophoresis, the following stock solutions and other solutions were prepared.

2.3.12.1 ò TAE buffer (p^H 8.3) (1 liter)

242 g Trizma Base (MW=121.14) was dissolved into 900 ml of sterile de-ionized distilled water. Then, 57 ml glacial acetic acid was added to the solution. Finally, 100 ml 0.5 EDTA (p^H 8.0) was added to it. These components were mixed well. The p^H of the solution was adjusted by mixing concentrated HCl at pH 8.3. The final volume of the solution was adjusted to 1000 ml.

2.3.12.2 6òLoading dye

Preparation of stock solutions

a. 10 ml of a 2% bromophenol blue stock solution.

b. 10 ml of a 2% xylene cyanol stock solution.

c. 50% glycerol solution.

The stock solutions were diluted to prepare 10 ml of the final 6×Loading dye with the following component and concentrations:

d. 30% glycerol

e. 0.3% bromophenol blue

f. 0.3% xylene cyanol

The 6× loading dye solution can be stored indefinitely in the refrigerator. The bromophenol blue, xylene cyanol, and glycerol stock solutions can be stored indefinitely at room temperature. The 6× DNA loading dye was added to DNA samples to achieve a final dye concentration of 1× loading dye.

2.3.12.3 Ethidium bromide solution

For 1 ml solution, Ethidium Bromide of 10 mg was added to 1 ml of sterile de-ionized distilled water. It was then mixed by hand shaking. The solution was then transferred to a dark bottle and stored at room temperature.

2.3.13 Agarose Gel-electrophoresis

The standard method used to separate, identify and purify DNA fragments through electrophoresis was followed according to the method described below:

a. 1.0 gm of agarose was heated to melt into 100 ml of TAE buffer. Ethidium bromide was added to 8µl final concentration and poured into gel tray fixed with appropriate combs.

b. After the gel was solidified it was placed into gel-running kit containing 1×TAE buffer.

c. Digested bacterial DNA solutions were loaded with 6× gel loading dye and electrophoresis was continued until DNA fragments were separated well.

2.3.14 Agarose Gel electrophoresis of the amplified products

The amplified PCR products were separated and confirmed electrophoretically on 1% agarose gel. The gel was prepared using 1.0 g agarose powder containing 0.5 µg/ml ethidium bromide and100 ml 10×TAE buffer. Agarose gel electrophoresis was conducted in 10× TAE buffer at 80 Volts and 300 mA for 40 minutes. One molecular weight marker 1.0 kb Plus DNA ladder was electrophoresed along side the reaction samples.

2.3.15 Documentation of the DNA sample

After completion of the electrophoresis, the gel was taken out carefully from the electrophoresis chamber and placed in Gel Documentation System (CSL-UVTS312, Cleaver Scientific LTD, UK) for checking the DNA bands. The DNA was observed as Band and photographe was taken using Gel Documentation system.

2.3.16 Purification of PCR product

Purification of PCR product was done using a kit (GeneJET^{TM}, Fermentas). At first, 25 µl of binding buffer was added to 25 µl of PCR product. The solution was transferred to the GeneJET^{TM} purification column and centrifuged at 13,000 rpm for 60 seconds. The flow through was then discarded and kept the DNA binding pellet with the filter through the

purification column. Then, washing buffer was added to the GeneJET^{TM} purification column and centrifuged at 13,000 rpm for 60 seconds. Then, the flow through was discarded and the purification column was placed back into the collection tube. The empty GeneJET^{TM} purification column was centrifuged for an additional 1 min to through away the residual wash buffer completely.

The GeneJET^{TM} purification column was transferred to the 1.5 ml sterile micro centrifuge tube. Then, 50 µl of elution buffer was added to the center of purification column membrane and centrifuged at 13,000 rpm for 1 minute. Finally the purification column was discarded and the purified DNA was stored at - 20°C.

2.3.17 Determination of the concentration of purified PCR product

For the determination of the concentration of PCR DNA, at first the spectrophotometer reading was made 0 with autoclaved water. Then, concentration of DNA was measured one by one. Measurements of absorbance were done at 260 and 280 nm. The 260:280 nm ratio was calculated. The ratio falling between 1.7 and 1.9 indicated pure solution of double-stranded DNA.

2.3.18 Sequencing and identification of bacteria

The purified PCR DNAs were sent to the CARS (Centre for Advance Research in Sciences) at University of Dhaka for sequencing. The volume of purified PCR product was 10 µl which was sent to the CARS. Along with purified PCR product, product size, concentration of the samples, a gel picture of purified PCR product, annealing temperature, volume of primer, final volume of PCR were written in a paper and sent to

the CARS. 10 µl working primer per reaction was also sent with purified PCR DNAs. The 3130 genetic analyzer was used for the sequencing. DNA sequencing was done using both forward and reverse primers, however, sequences obtained from forward primers were presented in this thesis.

2.3.19 Data analysis

Nucleic acid concentration was determined by using spectrophotometer. NCBI (National Centre for Biotechnology Information) BLAST (Basic Local Alignment Test) was used to identify the bacteria. Phylogenetic tree was constructed by using the software Phylogeny.fr.

3. RESULTS

3.1 Effects of salinity on the growth and development of lentil plants

3.1.1 Effects of salinity on shoot growth parameters

Effects of salt content on the shoot growth parameters such as shoot height, shoot fresh weight, shoot dry weight and leaf chlorophyll are shown in (Appendix 1). Appendix 1 shows that shoot height (cm) of the lentil variety that survived after salt application in the field, did not differed significantly ($F=1.86$, $P=0.21$) among different concentration of salts. Compared with control (0 mM), shoot height of the variety significantly decreased with the increase of salt treatment. Highest shoot height of the survived cultivar (14.30±0.99) was found in control (0 mM) but the lowest shoot height of the survived plants (11.45±1.37) was found in 100 mM salt treatment (Fig. 3.1).

Compared with control (0 mM), shoot fresh weight (g) of the survived plant after salt application did not differ significantly ($F=2.37$, $P=0.15$) among different concentration of salts. Highest shoot fresh weight (0.33±0.003) was found in 100 mM salt treatment. On the other hand, lowest shoot fresh weight (0.25±0.02) was found in 200 mM salt treatment (Fig. 3.2).

Shoot dry weight (g) did not differ significantly ($F= 1.97$, $P= 0.19$) among different concentration of salts. The highest shoot dry weight (0.11±0.009) was found in 100 mM

salt treatment. However, lowest shoot dry weight (0.08±0.003) was found in 50 mM (Fig. 3.3).

Chlorophyll content also did not differ significantly (F=0.85, P= 0.50) among different concentration of salts. However, chlorophyll content of lentil, under different salt concentrations, gradually decreased with the increase of salt treatment. The highest chlorophyll content (17.27±3.87 µg/cm^2) was found in control (NaCl free) and the lowest chlorophyll content (11.53±1.67 µg/cm^2) was found in 200 mM salt treatment (Fig. 3.4).

3.1.2 Effects of salinity on root growth parameters

Effects of salt content on the root growth parameters of lentil (BARI masur-4) are shown in Appendix 1. Appendix 1 shows that the root heights (cm) did not differ significantly (F= 1.01 P= 0.44) under different concentration of salts. However, highest root length (9.59±0.15) was found in control (0 mM) and the lowest root length (8.27±0.41) was found in 200 mM salt treatments (Fig. 3.5). Compared with control (0 mM), root fresh weight gradually decreased with the increase of salt treatment. However, root fresh weight (g) did not differ significantly (F= 0.46, P = 0.72) among different concentration of salts. Highest root fresh weight (0.10±0.01) was found in 100 mM and the lowest root fresh weight (0.09±0.01) was found in 200 mM salt treatment (Fig. 3.6).

Compared with control (0 mM), root dry weight decreased with the increase of salt treatment. However, root dry weight (g) did not differ significantly (F=1.0, P =0.44) among different concentration of salts. Highest root dry weight (0.01±0.00) were found

in 0 mM, 50 mM , 100 mM and the lowest root dry weight (0.009±0.00) was found in 200 mM salt treatment (Fig. 3.7).

As concentration salt increased root-to-shoot ratio of BARI masur-4 increased than control. Lowest root-to-shoot ratio was found in control (0 mM) and highest root-to-shoot ratio was found in 50 mM and 100 mM salt treatment (Fig. 3.8).

Salinity reduced the total nodule number per plant significantly. Nodule number per plant differed significantly (F=9.85, P=0.005) among different concentrations of salts. Highest number of nodule (1.30±0.01) was found in 50 mM and the lowest number of nodule (0.79±0.03) was found in 200 mM salt treatment (Fig. 3.9).

3.1.3 Proline

Effect of salt treatments on the proline concentration is shown in Appendix 2. Proline concentration increased with the increase of salt concentrations. The amount of proline was significantly affected by different salt concentrations (P = 0.01). In salt stress condition the highest amount of proline was found in 200 mM concentration (0.210±0.019) and lowest was found in 0 mM concentration (0.042±0.011) (Fig. 3.10).

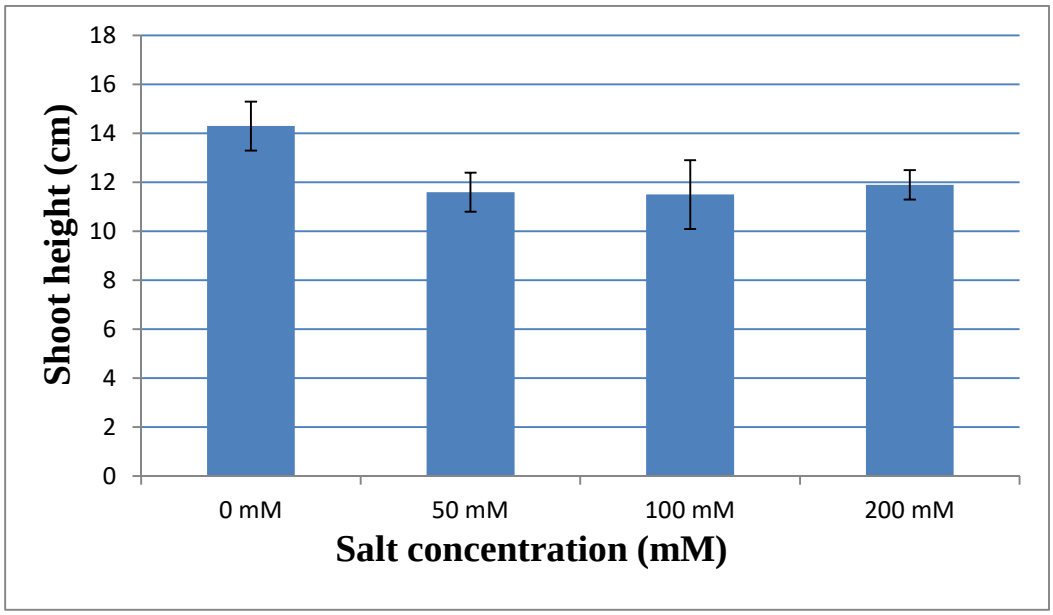

Fig. 3.1. Shoot height (mean ± SEM) of *Lens culinaris* Medik. var. BARI masur-4 plants treated with varying concentrations (0 mM, 50 mM,100 mM and 200 mM) of NaCl.

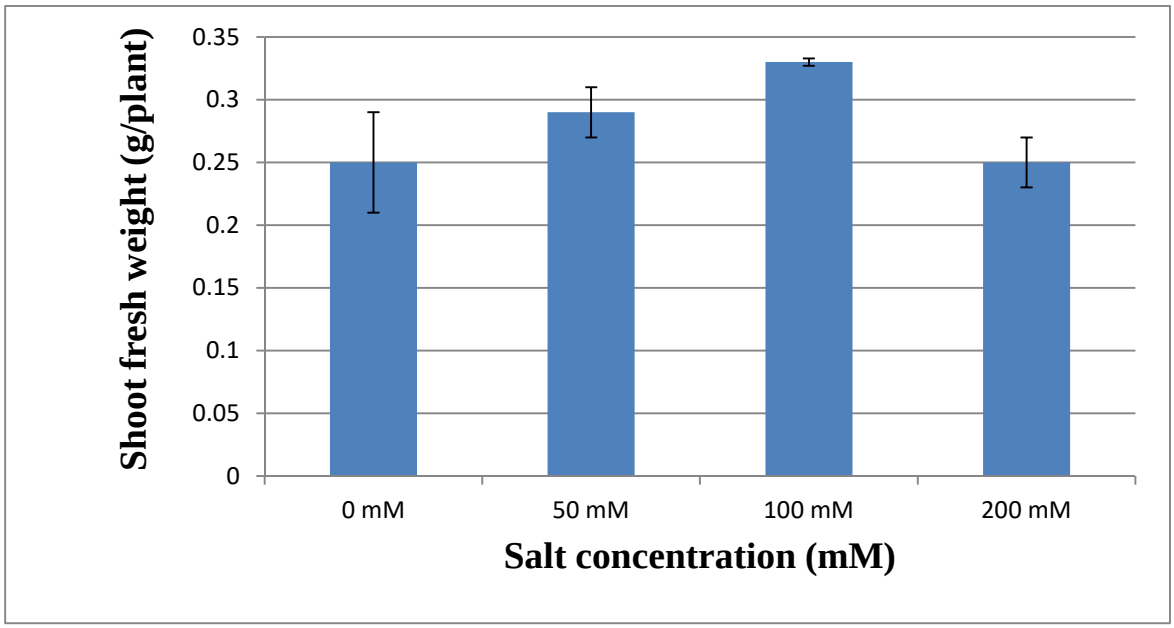

Fig. 3.2. Shoot fresh weight (mean ± SEM) of *Lens culinaris* Medik.var. BARI masur-4 plants treated with varying concentrations (0 mM, 50 mM, 100 mM and 200 mM) of NaCl.

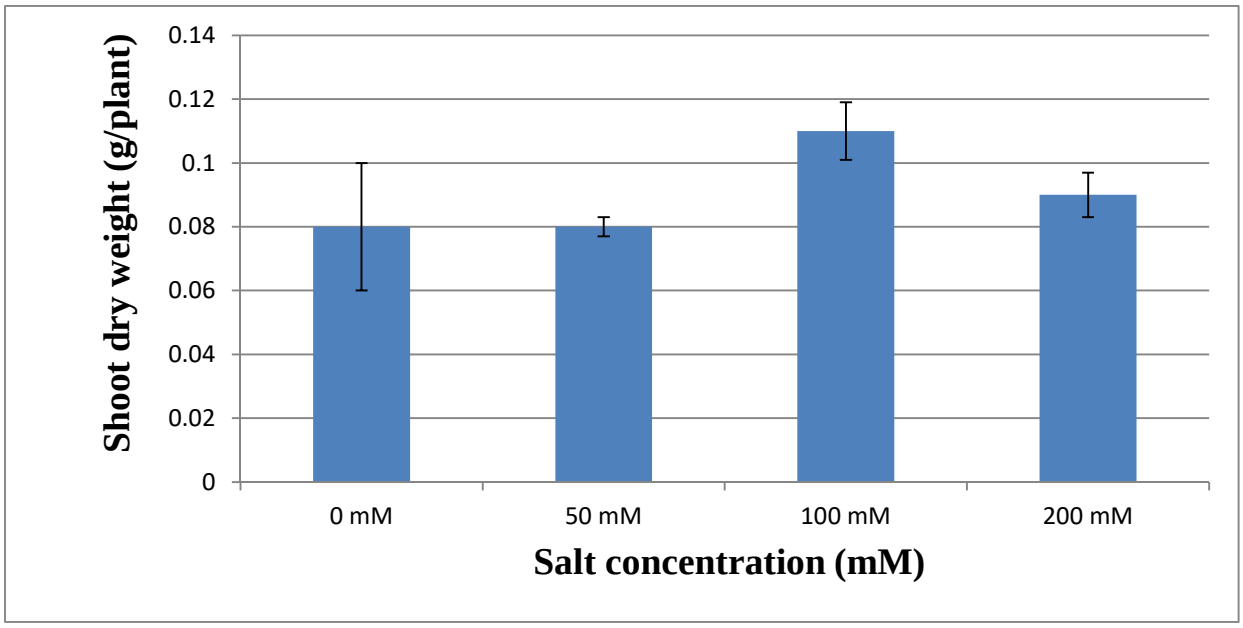

Fig. 3.3. Shoot dry weight (mean ± SEM) of *Lens culinaris* Medik. var. BARI masur-4 plants treated with varying concentrations (0 mM, 50 mM,100 mM and 200 mM) of NaCl.

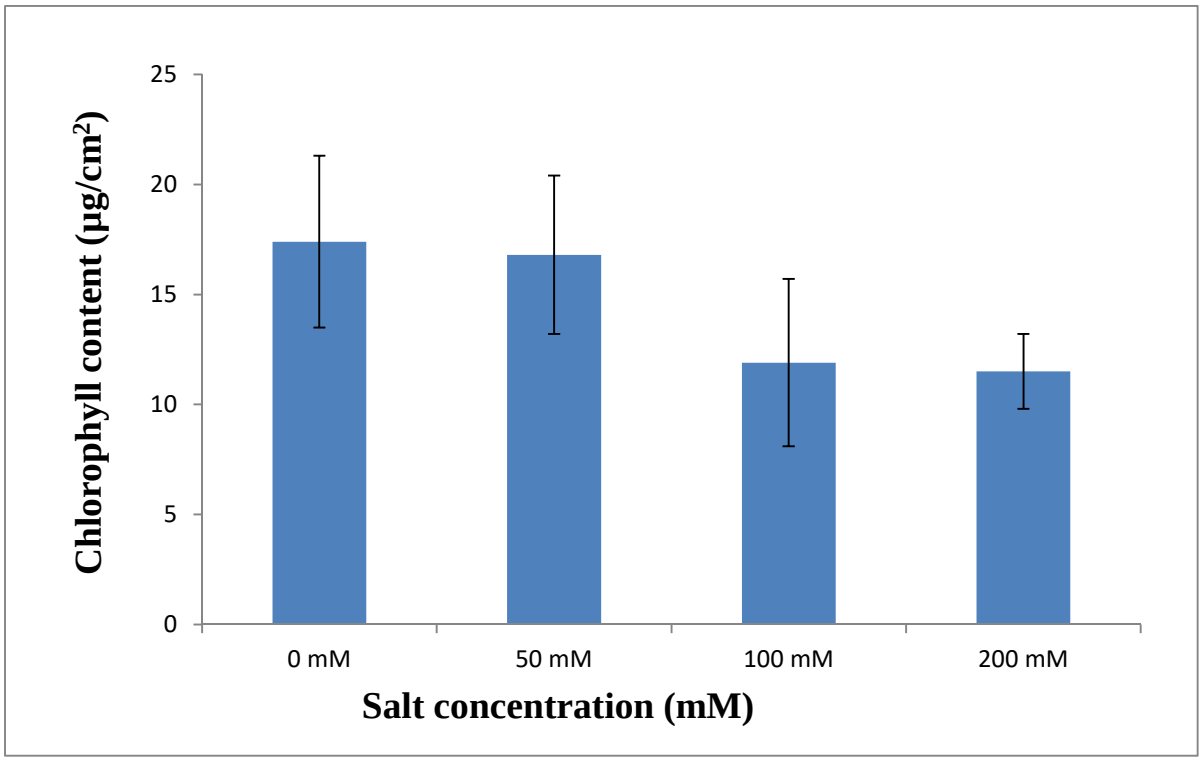

Fig. 3.4. Chlorophyll content (mean ± SEM) of *Lens culinaris* Medik.var. BARI masur-4 plants treated with varying concentrations (0 mM, 50 mM,100 mM and 200 mM) of NaCl.

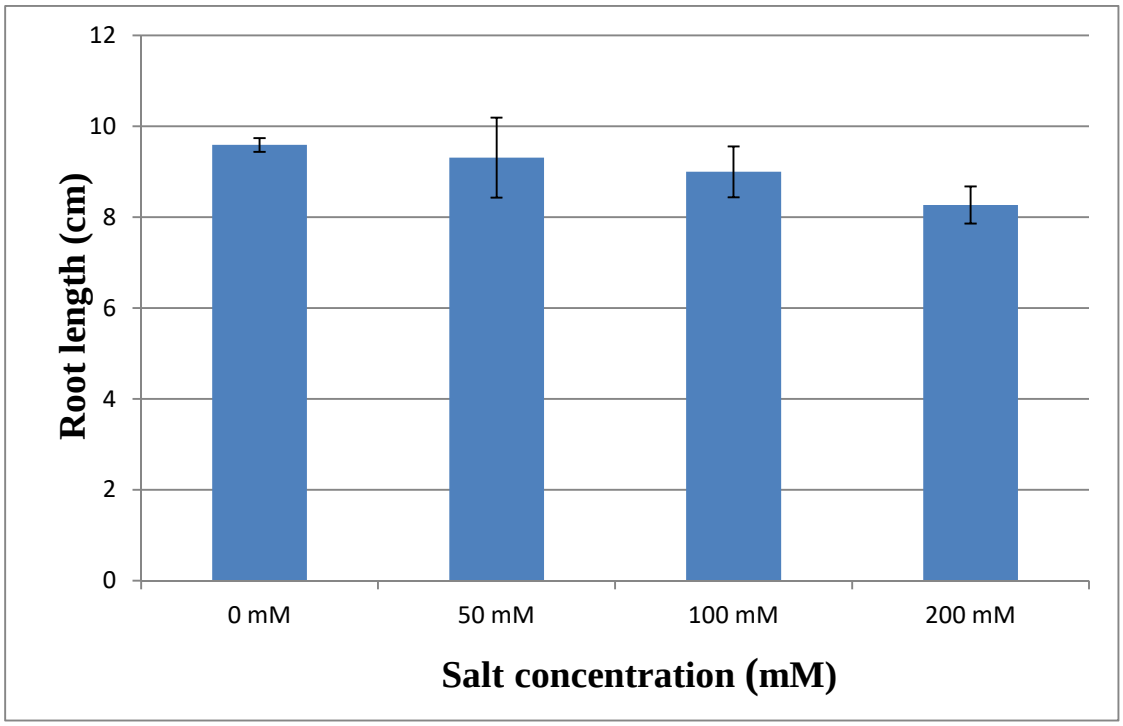

Fig. 3.5. Root length (mean ± SEM) of *Lens culinaris* Medik. var. BARI masur-4 plants treated with varying concentrations (0 mM, 50 mM,100 mM and 200 mM) of NaCl.

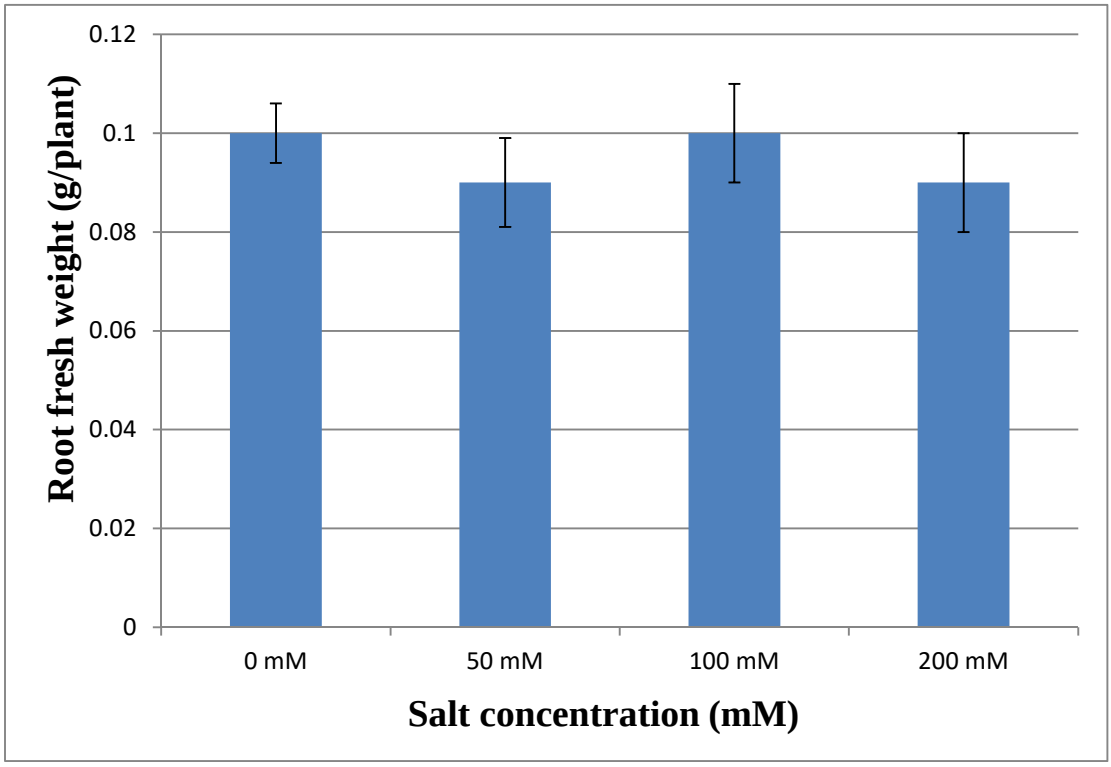

Fig. 3.6. Root fresh weight (mean ± SEM) of *Lens culinaris* Medik. var. BARI masur-4 plants treated with varying concentrations (0 mM, 50 mM,100 mM and 200 mM) of NaCl.

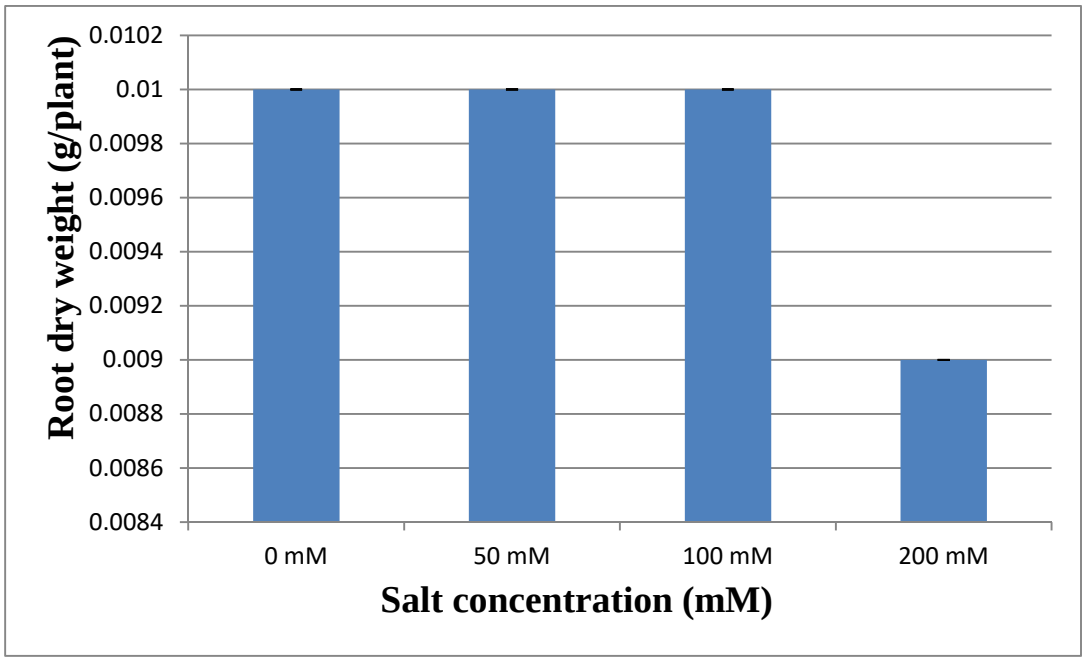

Fig. 3.7. Root dry weight (mean ± SEM) of *Lens culinaris* Medik. var. BARI masur-4 plants treated with varying concentrations (0 mM, 50 mM,100 mM and 200 mM) of NaCl.

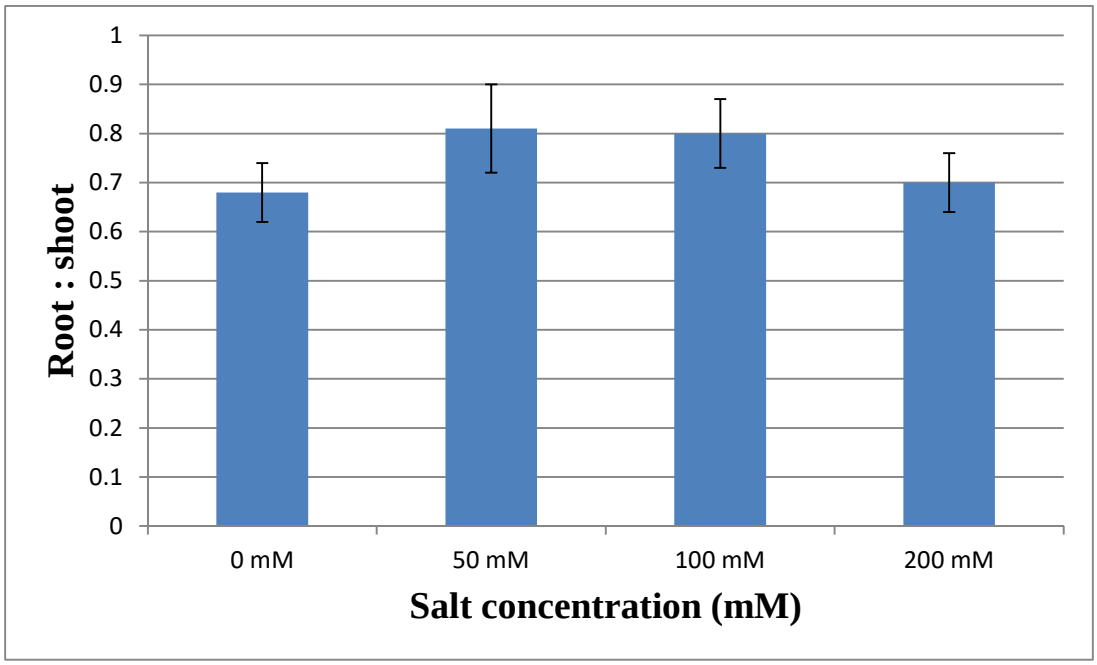

Fig. 3.8. Root:Shoot (mean ± SEM) of *Lens culinaris* Medik. var. BARI masur-4 plants treated with varying concentrations (0 mM, 50 mM,100 mM and 200 mM) of NaCl.

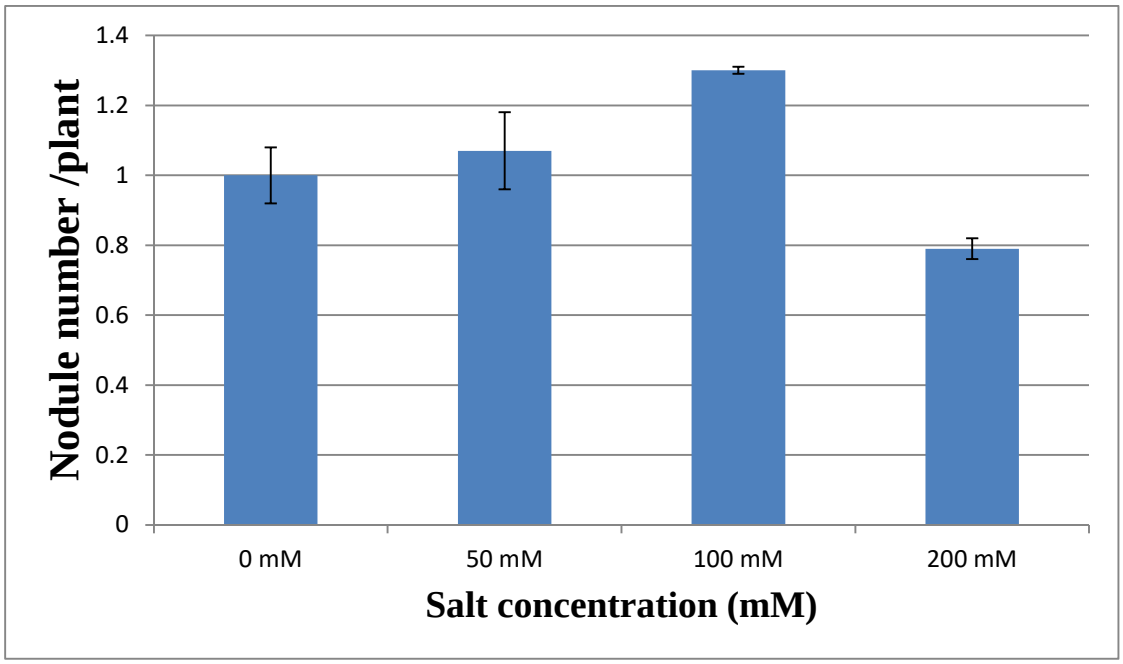

Fig. 3.9. Nodule number per plant (mean ± SEM) of *Lens culinaris* Medik.var. BARI masur-4 plants treated with varying concentrations (0 mM, 50 mM,100 mM and 200 mM) of NaCl.

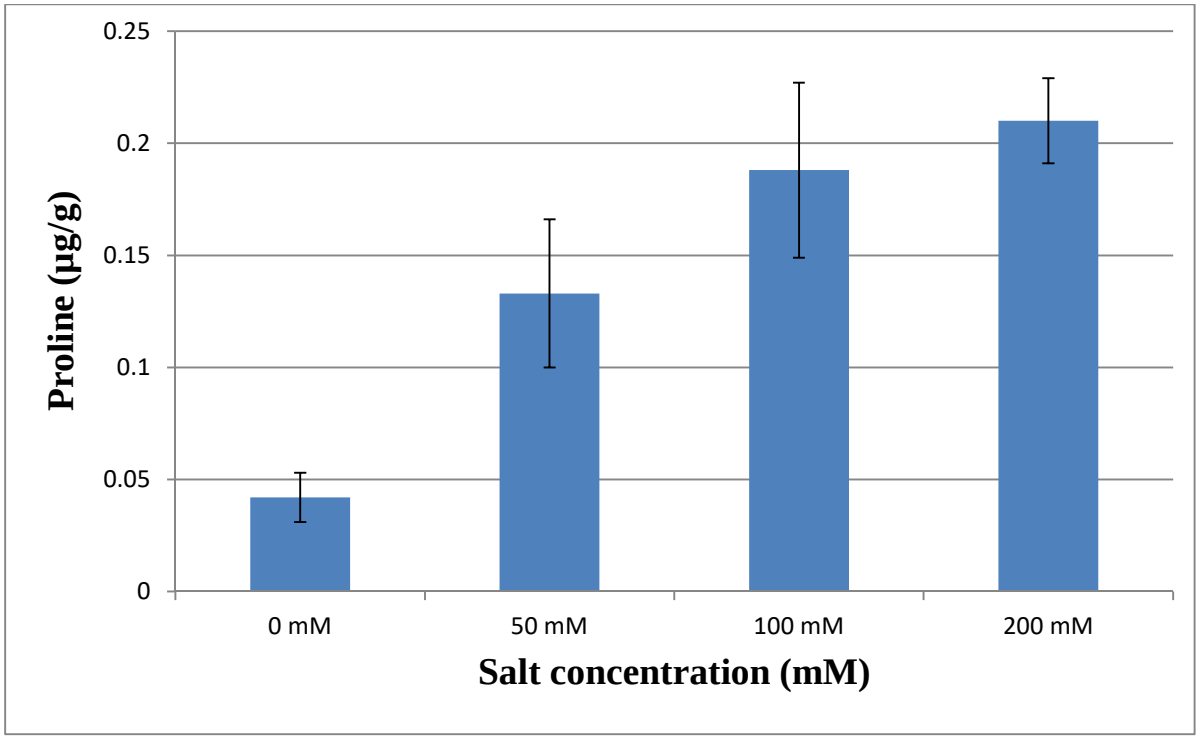

Fig. 3.10. Proline content (mean ± SEM) of *Lens culinaris* Medik.var. BARI masur-4 plants treated with varying concentrations (0 mM, 50 mM,100 mM and 200 mM) of NaCl.

3.2 Effects of salinity on bacterial the population associated with roots of lentil

3.2.1 Effects of salt concentrations on the bacterial colonies in root nodule

In this study, colonies of different size i.e. the small, medium and large size were examined in root nodules. The colonies were circular, light pink, convex, entire and opaque in morphology (Fig. 3.11). The number of small colony of bacteria found from root nodule did not differ significantly ($F=2.42$, $P=0.14$) among different concentrations of salt (Fig. 3.12). The highest number of small colony (491.67±39.1) was found in 100 mM concentration of salts and the lowest number of small colony (211.81±102.34) was found in control (without NaCl).

The number of medium size colony of bacteria obtained from root nodule differed significantly ($F=4.38$, $P=0.04$) among different salt treatments. Medium colony significantly increased with the increase of salt concentrations (Fig. 3.13). The highest number of medium colony (17.78±7.10) was found in 200 mM concentration of salts and the lowest number of medium colony (0.00±0.00) was found in control (without NaCl).

The number of large colony of bacteria obtained from root nodule differed significantly ($F=4.38$, $P=0.04$) among different salt concentrations. Large colony significantly increased with the increase of salt concentrations (Fig. 3.14). The highest number of large colony (29.44±0.68) was found in 200 mM concentration of salts and the lowest number of colony (0.00±0.00) was found in control (without NaCl).

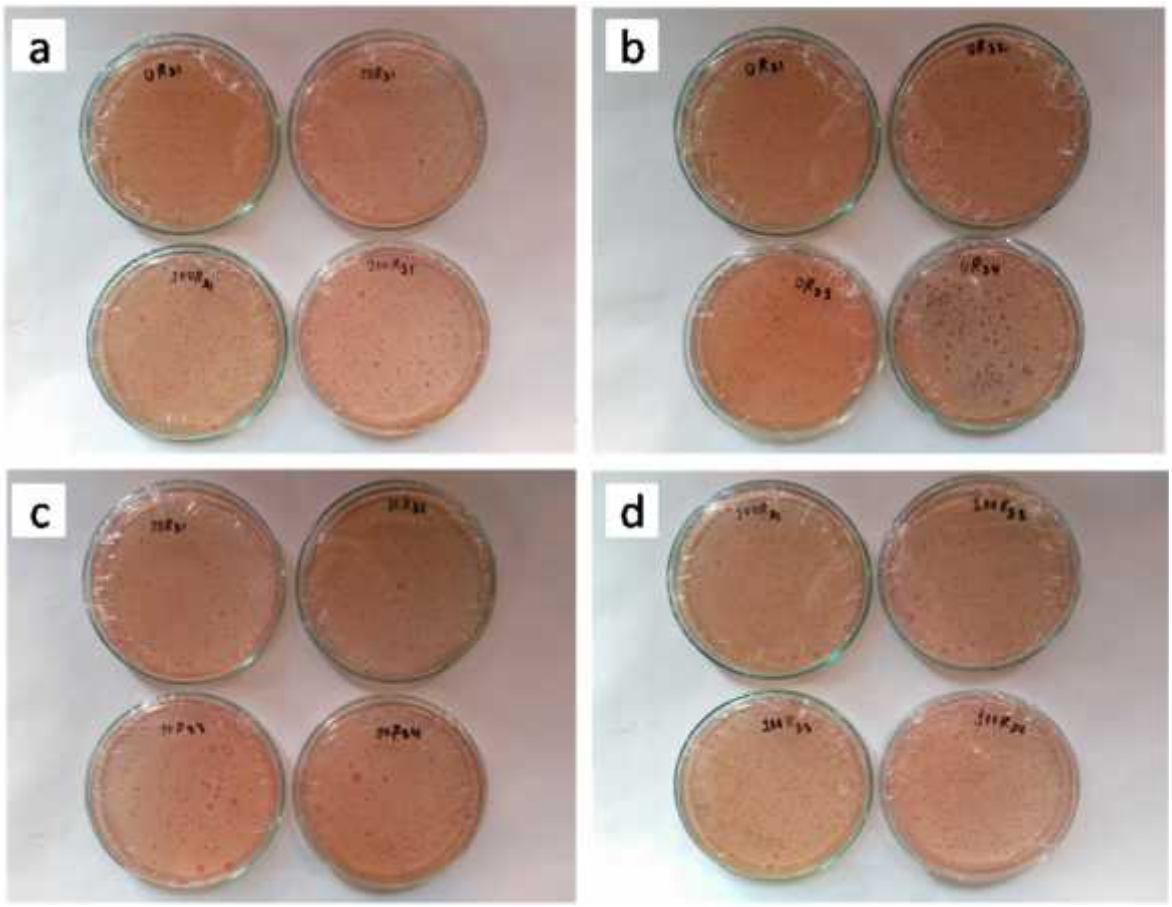

Fig. 3.11. Plates showing bacterial colony cultured from root nodule of lentil plants treated with varying concentrations (0 mM, 50 mM, 100 mM and 200 mM) of NaCl.

3.2.2 Number and size of bacterial colonies obtained from rhizosphere and bulk soil

Different size and number of bacterial colony were found from rhizosphere soil and the bulk soil at different concentration of salts. The colonies were of different sites; the small, medium and large size colonies. The colonies were circular, light pink, convex, entire and opaque in morphology.

There was no significant difference in the number of small colony observed between rhizosphere soil and bulk soil (Table 3.2). Small bacterial colony of the rhizosphere soil and bulk soil after 24 hours of incubation is shown in Fig. 3.15. Small colony of rhizosphere soil was highest in 100 mM concentration (213.33±92.72) and lowest in 0 mM concentration (78.75±34.92). But in case of bulk soil highest small colony was found in 200 mM concentration (180.42±25.36) and the lowest colony was found in 50 mM concentration. The highest number of small colony was found in rhizosphere soil than the bulk soil under different salt concentrations except control (without salt).

No significant difference of medium colony was observed between rhizosphere soil and bulk soil. Bacterial medium colony of the rhizosphere soil and bulk soil after 24 hours of incubation is shown in Fig. 3.16. Medium colony of rhizosphere soil was highest in 50 mM concentration (5.92±5.18) and lowest in 100 mM concentration (3.58±3.09). But in case of bulk soil, highest number of small colony was found in 50 mM concentration (4.83±4.83) and the lowest colony was found in 0 mM and 200 mM concentrations (0.17±0.08). The highest number of medium colony was found in rhizosphere soil compared with the bulk soil under different salt concentrations (Fig. 3.16).

There was significant difference in the number of large colony observed between rhizosphere soil and bulk soil. Bacterial large colony of the rhizosphere soil and bulk soil after 24 hours of incubation is shown in Fig. 3.17. Large colony of rhizosphere soil was highest in 50 mM concentration (7.83±5.88) and lowest in100 mM concentration (4.33±3.61). But in case of bulk soil highest number of large colony was found in 0 mM concentration (0.83±0.83) and the lowest colony was found in 50 mM concentration. The highest number of large colony was found in rhizosphere soil than the bulk soil under different salt concentrations.

Table 3.1. Mean values with standard error mean of the number of different colonies (Small colony, medium colony and large colony) from rhizosphere soil and bulk soil at different concentrations of NaCl (0 mM, 50 mM, 100 mM and 200 mM) after 24 hours.

NaCl Concentrations (mM)	Small colony		Medium colony		Large colony	
	Rhizosphere soil	Bulk soil	Rhizosphere soil	Bulk soil	Rhizosphere soil	Bulk soil
0	78.75±34.92	176.83±68.38	4.17±4.17	0.17±0.08	5.67±5.29	0.83±0.83
50	162.17±88.44	118.08±28.04	5.92±5.18	4.83±4.83	7.83±5.88	0.00±0.00
100	213.33±92.72	177.92±90.93	3.58±3.09	0.25±0.25	4.33±3.61	0.67±0.36
200	210.83±71.86	180.42±25.36	5.67±4.04	0.17±0.17	6.08±3.91	0.08±0.08

Table 3.2. Two-way ANOVA statistics on the effects of rhizosphere soil and bulk soil on different sizes of bacterial colonies (Small colony, medium colony and large colony) after 24 hours of incubation.

Source of variation	Small colony		Medium colony		Large colony	
	F-ratio	P value	F-ratio	P value	F-ratio	P value
Soil type	0.004	0.95	2.07	0.17	5.44	0.03
Concentration	0.56	0.65	0.43	0.74	0.06	0.98
Soil type ×Concentration	0.49	0.69	0.14	0.93	0.14	0.94

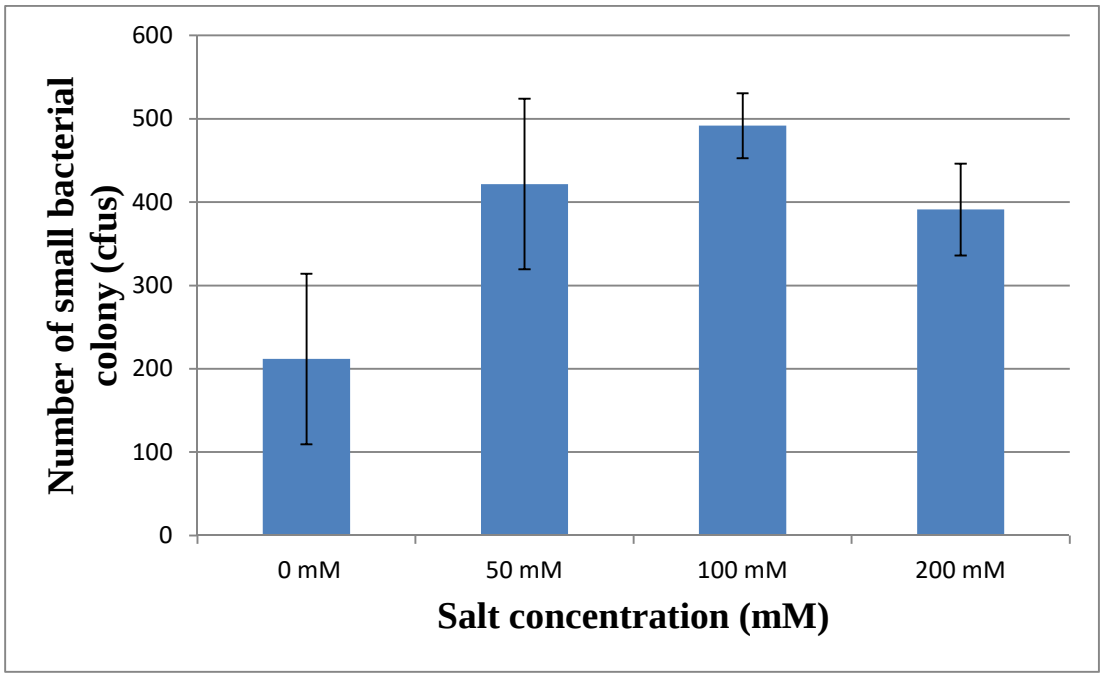

Fig. 3.12. Number of small bacterial colony (mean ± SEM) cultured from root nodule of *Lens culinaris* Medik.var. BARI masur-4 plants treated with varying concentrations (0 mM, 50 mM,100 mM and 200 mM) of NaCl.

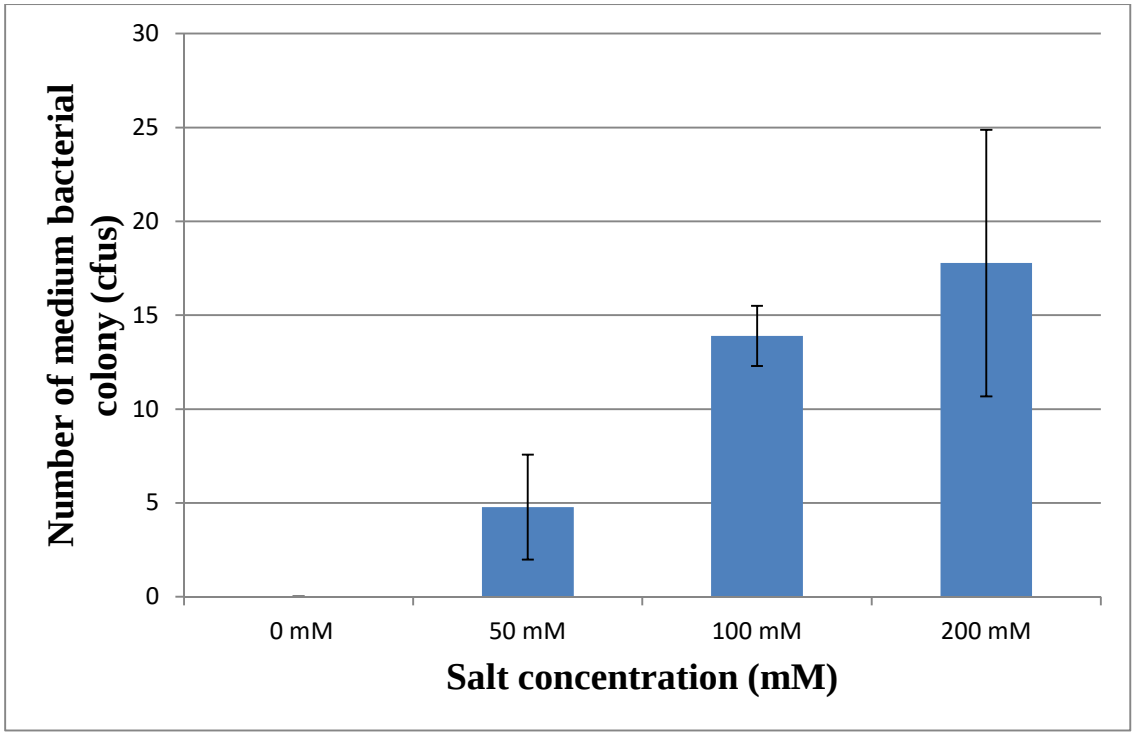

Fig. 3.13. Number of medium bacterial colony (mean ± SEM) cultured from root nodule of *Lens culinaris* Medik.var. BARI masur-4 plants treated with varying concentrations (0 mM, 50 mM,100 mM and 200 mM) of NaCl.

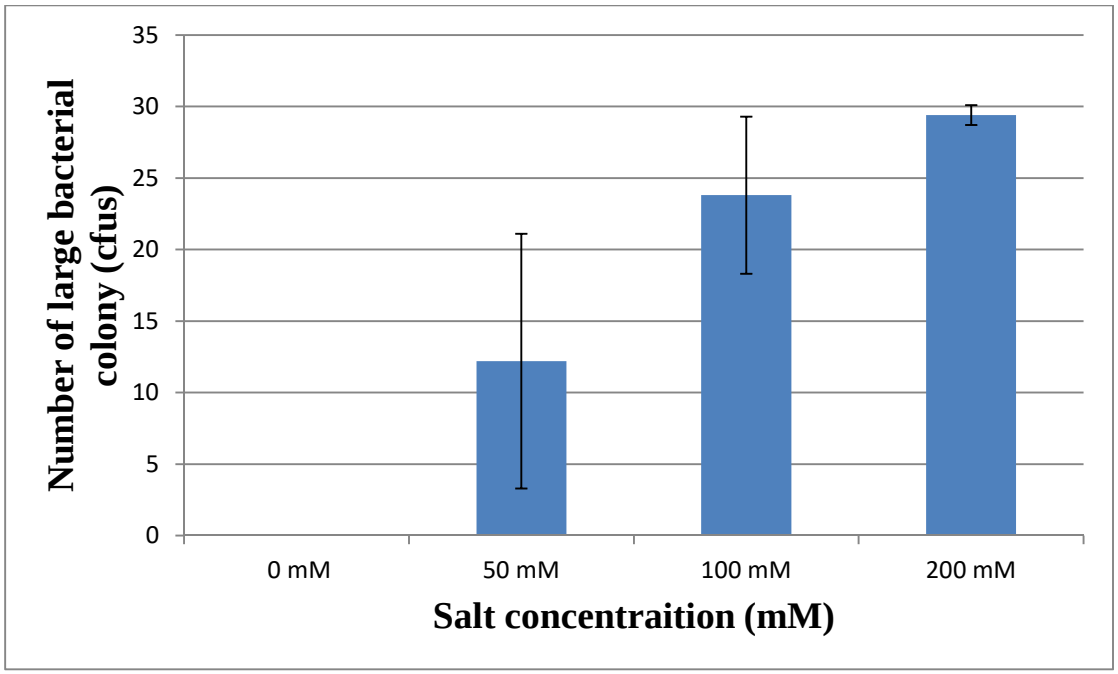

Fig. 3.14. Number of large bacterial colony (mean ± SEM) cultured from root nodule of *Lens culinaris* Medik.var. BARI masur-4 plants treated with varying concentrations (0 mM, 50 mM, 100 mM and 200 mM) of NaCl.

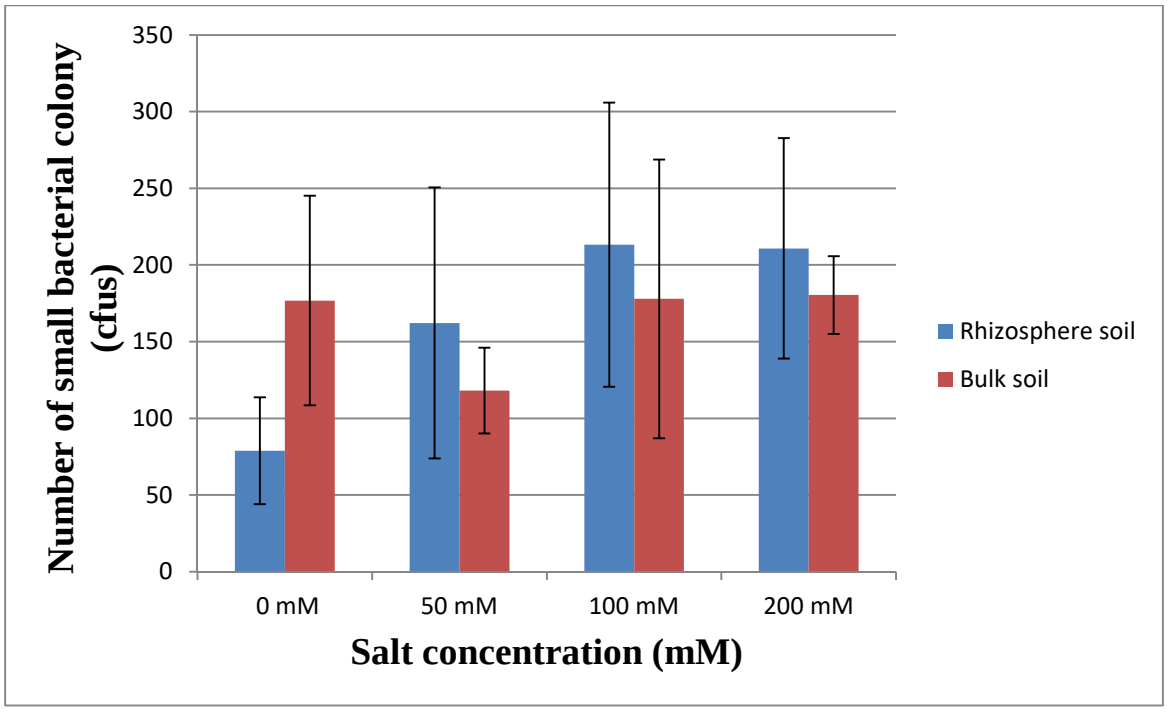

Fig. 3.15. Number of small bacterial colony (mean ± SEM) cultured from rhizosphere soil and bulk soil under *Lens culinaris* Medik.var. BARI masur-4 treated with varying concentrations (0 mM, 50 mM,100 mM and 200 mM) of NaCl.

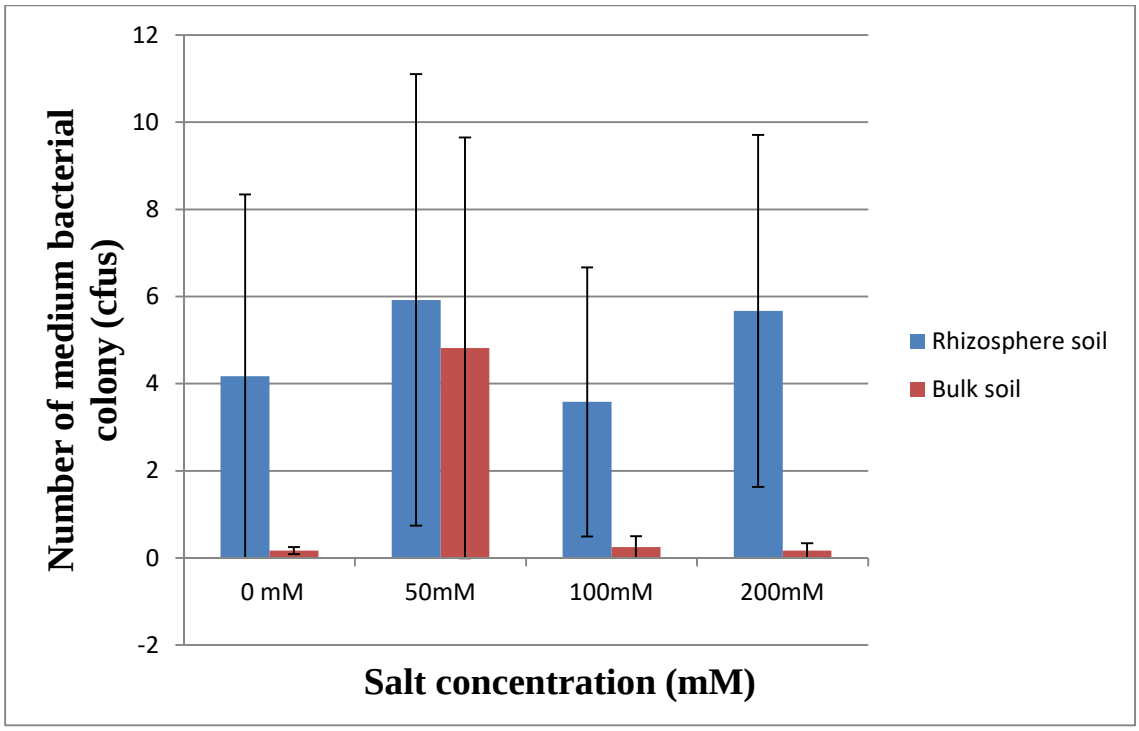

Fig. 3.16. Number of medium bacterial colony (mean ± SEM) cultured from rhizosphere soil and bulk soil under *Lens culinaris* Medik.var. BARI masur-4 treated with varying concentrations (0 mM, 50 mM, 100 mM and 200 mM) of NaCl.

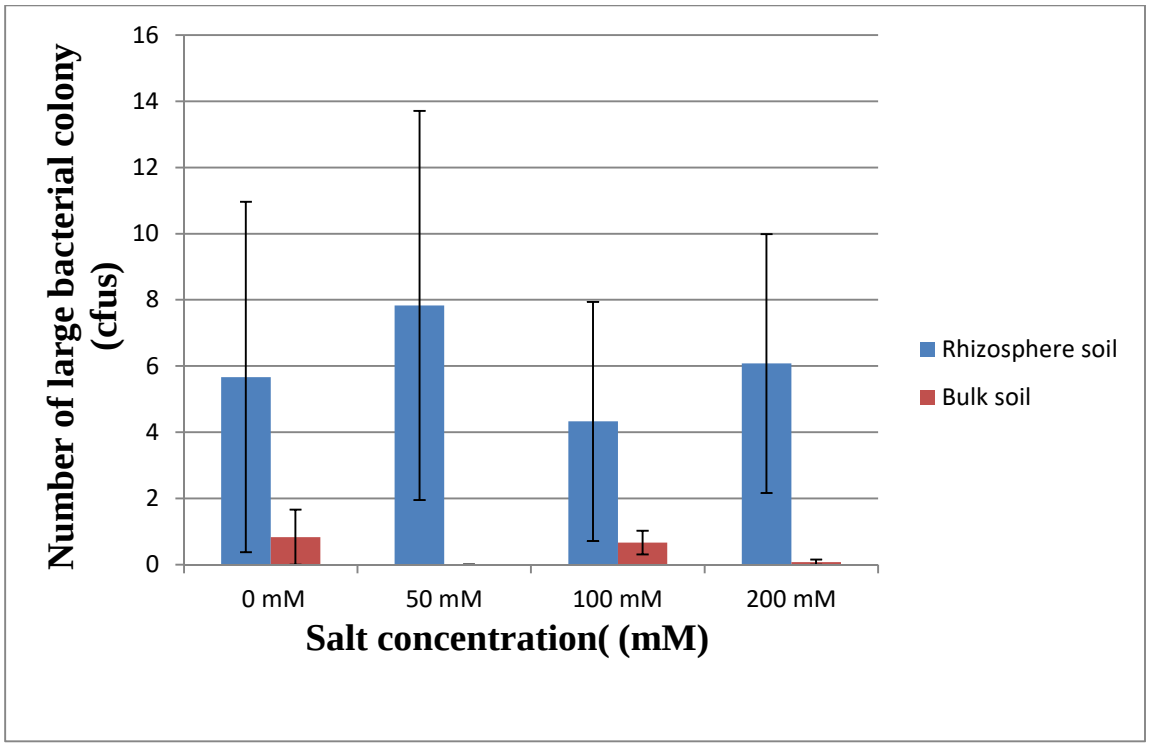

Fig. 3.17. Number of large bacterial colony (mean ± SEM) cultured from rhizosphere soil and bulk soil under *Lens culinaris* Medik.var. BARI masur-4 treated with varying concentrations (0 mM, 50 mM, 100 mM and 200 mM) of NaCl.

3.3 Identity and genetic diversity of *Rhizobium*

In this study, the compatible nitrogen fixing bacteria *Rhizobium* (*Rhizobium leguminosarum)* was identified by using 16s rRNA gene. Nucleic acid concentration was estimated which showed variation from sample to sample is shown in table 3.3.In this study ,16s rRNA gene was sequenced using both forward and reverse primers.

Table 3.3. Estimation of nucleic acid concentrations (ng/µl) of the bacterial isolates.

Isolate ID	Concentration (ng/µl)	A260/A280	A260/A230
R-5	7.85	1.62	0.18
R-6	16.77	2.48	0.48
C-8	8.72	1.47	0.09
C-9	7.03	2.12	0.71

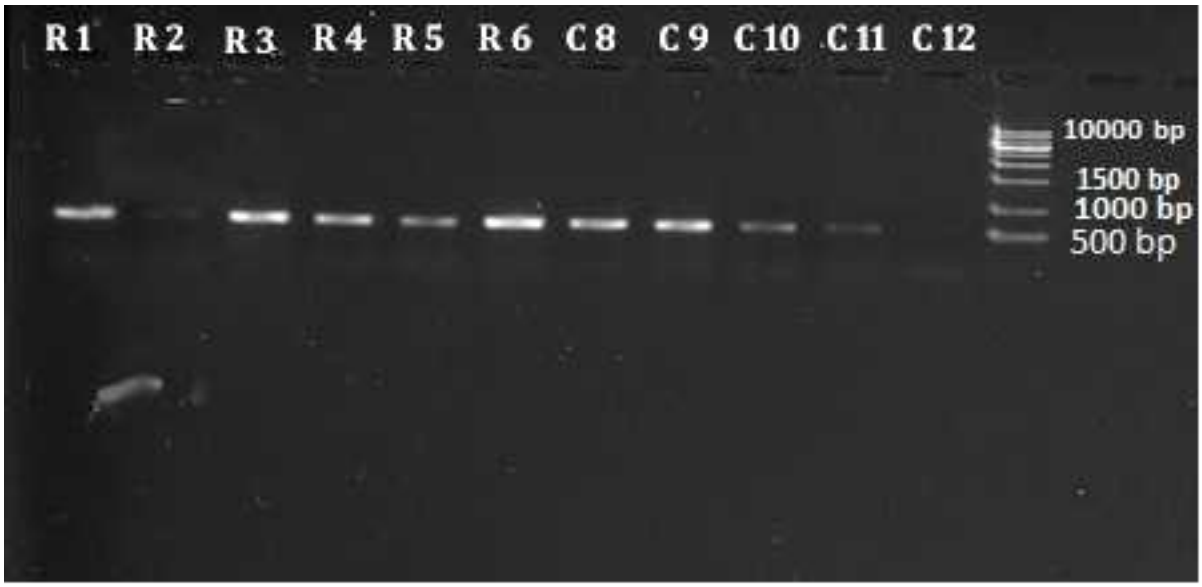

Fig. 3.18. Picture showing Gel electrophoresis. R1, R2, R3, R4, R5, R6 obtained from root nodule of Rajshahi area and C8, C9, C10, C11, C12 obtained from root nodule of Cumilla area.

Rhizobium was found only in Rajshahi and Cumilla; isolate R-5 and R-6 from Rajshahi and C-8 and C-9 from Cumilla. Two organisms was found from isolate R-5 and R-6 by using 16s rRNA primer. They were *Rhizobium* sp. Strain 42T and *Rhizobium leguminosarum* strain PB62. *Rhizobium* sp. Strain 42T found 97% identity similarity, E value 0.0 and Accession KX884983.2. *Rhizobium leguminosarum* strain PB62 found 97% identity similarity, E value 0.0 and Accession EF525210.1.

Similarly, Two organisms found from isolate C-8 and C-9 using 16s rRNA primer. They were *Rhizobium* sp. Strain P40RR-XXII, *Rhizobium* sp. Strain ESA 90. *Rhizobium* sp. Strain P40RR-XXII found 96% identity similarity, E value 0.0 and Accession MG786751.1. *Rhizobium* sp. Strain ESA 90 found 95% identity similarity, E value 0.0 and Accession MF288755.1.

Table 3.4. Bioinformatics of the DNA sequences obtained from rhizobium isolates using NCBI (National Centre for Biotechnology Information) BLAST.

Isolate Id	Organisms name	Max score	Total score	Query length	Query cover	E value	Ident	Accession
R-5	*Rhizobium* sp. Strain 42T	896	896	552	97%	0.0	97%	KX884983.2
R-6	*Rhizobium leguminosarum* strain PB62	667	667	459	86%	0.0	97%	EF525210.1
C-8	*Rhizobium* sp. Strain P40RR-XXII	712	712	486	90%	0.0	96%	MG786751.1
C-9	*Rhizobium* sp. Strain ESA 90	837	837	552	97%	0.0	95%	MF288755.1

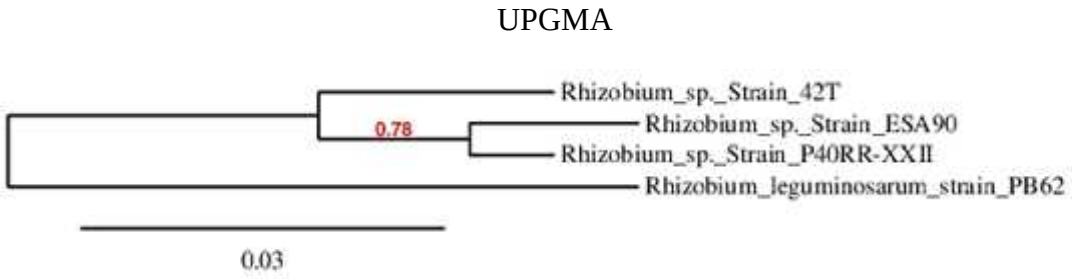

Fig. 3.19. Phylogenetic tree based on the 16S rRNA sequencing of isolated bacteria Phenogram demonstrating the relationships among the studied *Rhizobium* spp. This four isolates were collected from Rajshahi and Cumilla. Among these 4 strains, *Rhizobium* sp. strain 42T and *Rhizobium leguminosarum* strain PB62 were collected from Rajshahi area. *Rhizobium* sp. strain ESA90 and *Rhizobium* sp. strain P40RR-XXII collected from Cumilla area.

DNA sequences of the *Rhizobium* isolates obtained by using NCBI Blast online gene bank are shown in Appendix 7.The first cluster included *Rhizobium* sp. strain 42T, *Rhizobium* sp. strain ESA90 and *Rhizobium* sp. strain P40RR-XXII. Among these 3 strains of *Rhizobium*, *Rhizobium* sp. strain ESA90 and *Rhizobium* sp. strain P40RR-XXII although these two are not closely related to *Rhizobium* sp. strain 42T. However, the first cluster included *Rhizobium leguminosarum* strain PB62. These results indicate genetic diversity among the isolates obtained from the *Rhizobium.*

4. DISCUSSION

Soil salinity is a worldwide problem. It becomes a major issue in global agriculture. Salinity causes unfavorable environment and hydrological situation that restrict normal crop production. The coastal area covers about 20% of the total area of Bangladesh which is 147,570 km^2 and over thirty percent of the net cultivable area. The cultivable areas in coastal districts are affected with varying degrees of soil salinity. The coastal and offshore area of Bangladesh includes tidal, estuaries and river floodplains in the south along the Bay of Bengal. Agricultural land use in these areas is very poor, which is roughly 50% of the country's average (Petersen and Shireen 2001). Currently, new areas are also being affected by salinity in coastal districts and reducing the total cultivable area. Salinity is one of the environmental adverse conditions which is detrimental to plants. The main obstacle to intensification of crop production in the coastal areas is seasonally high content of salts in the root zone of the soil. Soil salinity is the most dominant limiting factor in the coastal region, especially during the dry season. It affects certain crops at different levels of soil salinity and at critical stages of growth, which reduces yield and in severe cases total yield is lost.

Lentil is an important legume plant because it provides high-quality protein for human consumption and its straw is highly valued animal fodder. The crop is salt sensitive (Ashraf and Waheed 1990) like many other leguminous crops to salt stress. It decreases the crop quality and yield by imposing toxic ion effect and water stress. Selection of salt-tolerant varieties is most important for cultivation on saline soils. Soil Salinity affects the nodulation in lentil plants where N fixation takes place (Tate

1995). Biological nitrogen fixation occurs through symbiotic association with legume and rhizobia which is also affected by salinity (van Hoorn *etal.* 2001). Fertility status of most saline soils ranges from low to very low in respect to organic matter content, nitrogen, phosphorus and micronutrients like zinc and copper. Rhizobia can fix atmospheric nitrogen to increase soil fertility. Among various environmental factors, salinity is one of the major factors that severely affect bacterial population, nodule formation and biological nitrogen fixation. Given this situation, the results of the present study is described with the emphasis on nodulation and rhizobial population in saline soils.

Morphologically, the most typical symptom of saline injury to a plant is retardation with growth parameter of shoot (shoot height, shoot fresh weight, shoot dry weight and shoot water content) (Bandeoglu *et al.* 2004). In this study, it was found that shoot height of lentil plants gradually decreased with the increased contents of salinity. Decreased growth under salt stress has been shown in many plant species. Kapoor and Srivastava (2010) reported decline in the lengths and height with the increased salt concentration in mungo bean (*Vigna mungo* L.). On the other hand, significant relationship was not found in shoot fresh weight and shoot dry weight. Memon *et al*. (2010) in their study on field mustard (*Brassica campestris* L.) showed that the use of low concentrations of sodium chloride led to the increases in plant lengths, whereas high concentrations caused reduced growth. Several studies showed that fresh and dry weights of the shoot systems are affected, either negatively or positively, by changes in salinity concentration, type of salt present, or type of plant species (Jimenez *et al.* 2002, Jamil *et al.* 2005).

In this study it was also found that chlorophyll content of lentil leaf decreased with the increase of salinity. The result of the present study agreed with that of Tort and Turkyilmaz (2004) who reported that the exposure of barley (*Hordeum vulgare* L.) to zero, 120, and 240 mM of sodium chloride led to the decrease in chlorophyll content. Siler *et al.* (2007)in their study also reported decreased chlorophyll content with the increase of salt concentrations in common centaury (*Centaurium erythraea* L.). However, no effect of salinity on the total chlorophyll content was also reported by some studies (e.g. Turhan and Eris 2005).

The present study showed that root height decreased by increase of salinity. It also showed that, root fresh and dry weight did not vary largely with the salt concentrations. Orak and Ates (2005) on common vetch (*Vicia sativa* L.), and Nedjimi *et al.* (2006), on Mediterranean saltbush (*Atriplex halimus* L.) reported an increase in fresh and dry weight for root and shoot systems of the plants with concentrations of NaCl. Yurtseven *et al.* (2005) reported that biomass decreased with increasing salinity levels in plants. Reduction in biomass with the increase of salt concentrations was reported by other investigations also (e.g. Netondo *et al.* 2004, Krishnamurthy *et al.* 2007). Since, in the present study, plants were grown in the field condition, other soil properties might have suppressed the negative effects of salt on the plant root growth (Hossain *et al*. 2016b).

The present study also showed that root to shoot ratio was not affected by salt treatments but the ratio increased with the increase of salt compared to control. Hafeez *et al.* (1988) has reported that salt stress may affect the infection process by inhibiting root hair growth and by decreasing the number of nodules per plant.

Reduced growth of root systems due to increased salt content can also be associated with the reduced number of nodule produced per plant. In this study it was observed that the number of nodule per plant decreased with the increase of salinity. Other studies also showed that the process of nodule initiation in plants is extremely sensitive to NaCl and that nodule number reduced with the increase of salt concentration (Singleton and Bohlool 1984, Elsheikh and Wood 1990).

Data of the present study showed that proline concentration increased significantly with the increase of salt concentrations. Other studies also reported high proline content in plant tissues under high salt concentration (Hossain *et al.* 2018, Bandeoğlu *et al*. 2004, Misra and Gupta 2005). Many other environmental stresses have also been reported to increase the level of proline in plants.Increase in proline concentration correlated with the increase in drought stress condition (Hossain *et al.* 2016a). These results indicate that the production of proline is a common response to various abiotic stresses. Proline can be regarded as an important osmoprotectant in plants and salt tolerance in plants, therefore, has often been attributed to the accumulation of osmoprotectants. Result of the present study is also in consistent with Kanawapee *et al.* (2013) who reported that under salt stress condition the highly susceptible cultivars accumulated the highest level of proline than the tolerant cultivars. Results thus indicate that over accumulation of proline can be related to a symptom of salt injury rather than an indicator of salt resistance (Lutts *et al.*1999).

Results of the present study also demonstrated that number of bacterial colonies across all sizes increased with the increase of concentrations of salt treatment in root nodule. It also showed that number of bacterial colonies generally increased with the

increase of salt concentrations in both rhizosphere and bulk soils. Moreover, number of bacterial colony was relatively higher in the rhizosphere soils compared to bulk soil across most of the salt treatments. Other study also found that bacterial populations are enriched in the rhizospheres of plants compared to the surrounding bulk soil (Smalla *et al*. 2001). Community composition of bacteria in rhizosphere differs from that in the bulk soil (Marilley and Aragno 1999). Root exudation as well as the structure of roots in rhizosphere might have influenced the bacterial copulation and composition in the rhizosphere. Although salt treatment might be detrimental to the bacterial growth and population through their inhibitory effect (Zahran1999) up to certain concentration, salt could enhance the growth of bacteria by providing nutrients to microbes. Yang *et al.*(2016) reported that high severity of salt stress increased abundance and diversity of bacterial communities in the rhizosphere soil of Jerusalem artichoke (*Helianthus tuberosus*). Salinity is one of the most important abiotic factors that affect the shaping of microbial community composition (Lozupone and Knight 2007). It has been reported that the root colonization is affected by salinity and also soil type (Compant *et al*. 2010). Salt tolerant and root colonizing bacteria can survive in harsh environment and help plant to tolerate salt stress (Ahmad *et al*. 2013, Egamberdieva *et al.* 2016).

The nodule forming bacteria *Rhizobium* (*Rhizobium leguminosarum)* compatible to lentil plants was also identified in the present study. Root nodule collected from different areas of the country and bacteria was isolated from the nodules. It indicates that nitrogen fixation of lentil plants occur with the association of *R. leguminosarum*. The result of phylogenetic tree also showed that *Rhizobium* spp. are genetically very much different based on the 16s rRNA sequencing of isolated bacteria. The clusters of

phylogenetic tree demonstrated the relationship among different *Rhizobium* spp. A number of previous studies have suggested that fast growing isolates are generally more salt-tolerant than slow growing isolates (Graham and Parker 1964, Bala *et al*. 1990, Marsudi *et al*. 1999, Mohamed *et al*. 2000, Yates *et al*. 2004). Barnet and Catt (1991) characterized the growth rates of *Acacia* rhizobia from both temperate and arid sites. Similar studies of West Australian native legumes (including *Acacia* spp.) have shown that fast-growing *Rhizobium* were able to survive and grow in 170 mM NaCl and also at 37 °C, whereas slow-growing *Bradyrhizobium* were less viable under these conditions (Marsudi *et al.* 1999, Yates *et a*l. 2004). Among the 4 strains of *Rhizobium*, the strains identified from Cumilla samples belonged to the same cluster in the phylogenetic tree which indicated that the strains were more similar perhaps due to the similar ecological condition. On the other hand, the strains identified from Rajshahi showed large separation from each other as well as from the cluster of Cumilla strains which indicate that origin of these two strains perhaps were separate.

Overall, the results of the present study indicate that salinity have strong effects on both vegetative growth and physiological processes in lentil plant. Shoot height, shoot fresh weight, shoot dry weight, root height, root fresh weight and root dry weight of lentil plants were reduced with high salt treatment. All these results also indicate that plants have a strong influence on the microbial populations on their roots. Results further demonstrate that salt can influence the growth of lentil plant, root nodule number as well as bacteria present in the root nodules although the effects vary with the concentration of salts. However, although the number of bacterial colony increased with the increase of salt content, total number of nodule per plant is significantly reduced indicating that net biological fixation of nitrogen might be

reduced in the high salinity condition in soil. However, it is not investigated whether the identified nodule forming rhizobia are salt tolerant or not. Therefore future study is needed for identifying salt resistant or tolerant rhizobia for better management of agricultural soil affected by salinity.

5. REFERENCES

Afzal MA, Bakr MA and Rahman ML 1999.Lentil cultivation in Bangladesh.Lentil, Black gram and Mung Bean Development Pilot Project, Pulses Research Station, BARI, Gazipur.

Ahmad P 2010. Growth and antioxidant responses in mustard (*Brassica juncea* L.) plants subjected to combined effect of gibberellic acid and salinity. Arch. Agron. Soil Sci.**56**(5): 575-588.

Ahmad P and Prasad MNV 2012. Abiotic stress responses in plants: metabolism, productivity and sustainability, Springer, New York, NY, USA.

Ahmad P and Umar S 2011. Oxidative stress: Role of antioxidants in plants, Studium Press, New Delhi, India.

Ahmad M, Zahir ZA, Khalid M, Nazli F and Arshad M 2013. Efficacy of *Rhizobium* and *Pseudomonas* strains to improve physiology, ionic balance and quality of mung bean under salt-affected conditions on farmer's fields. Plant Phys. Biochemistry **63**: 170-176.

Ali AMS 2006. Rice to Shrimp: Land Use/Land Cover Changes and Soil Degradation in Southwestern Bangladesh. Land Use Policy **23**(4): 421-435.

Angle JS, Levin MA, Gagliardi JV,McIntosh MS . 1996 . Soil Biochem.Marcel Dekker, Inc., New York.

Apel K and Hirt H 2004. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu. Rev. Plant Biol. **55**:373-399.

Ashraf M and McNeilly T 2004.Salinity tolerance in Brassica oilseeds. Crit.Rev. Plant Science **23**: 157–174.

Ashraf M and Waheed A 1990.Screening of local-exotic accessions of lentil (*Lens Culinaris* Medik.) for salt tolerance at 2 growth stages. Plant and Soil **128** (2): 167-176.

Bala N, Sharma PK and Lakshminarayana K 1990.Nodulation and nitrogen fixation by salinity-tolerant rhizobia in symbiosis with tree legumes. Agric. Ecosyst. Environ. **33**: 33-46.

Bandeoğlu E, Eyidoan F, Yücel M and Oktem HA 2004. Antioxidant responses of shoots and roots of lentil to NaCl-salinity stress. Plant Gr. Regul. **42**(1): 69-77.

Barnet YM and Catt PC 1991.Distribution and characteristics of root-nodule bacteria isolated from Australian *Acacia* spp. Plant and Soil. **135**(1): 109-120.

Battacharya D, Rahman M and Khatun FA 1999. Environmental impacts of trade liberalisation and policies for the sustainable management of natural resources: A case study on Bangladesh shrimp farming industry, United Nations Environment Programme (UNEP), New York and Geneva.

BBS 1995.Yearbook of Agricultural Statistics of Bangladesh. Bangladesh Bureau of Statistics, Ministry of Planning, Government of the People's Republic of Bangladesh, Dhaka.

BBS 2009.Yearbook of Agricultural Statistics of Bangladesh. Bangladesh Bureau of Statistics, Ministry of Planning, Government of the Pepole's Republic of Bangladesh, Dhaka.

BBS 2016.Yearbook of Agricultural Statistics of Bangladesh. Bangladesh Bureau of Statistics, Ministry of Planning, Government of the Pepole's Republic of Bangladesh, Dhaka.

Beckmann J and Soller M 1993. Restriction fragment length polymorphism in genetic improvement methodology. Mapping and cost. Theor. Appl. Genet. **67**: 33–43.

Benlloch M, Ojeda MA, Ramos J and Rodr´ıguez-Navarro A 1994.Salt sensitivity and low discrimination between potassium and sodium in bean plants. Plant Soil **166**: 117–123.

Bornet BC, Muller FP and Branchard M 2002. Highly informative nature of inter simple sequence repeat (ISSR) sequences amplified using tri and tetra-nucleotide primers from DNA of cauliflower (*Brassica oleracea* var. 'botrytus' L.). Genome **45**: 890-896.

Brantestam AK, Bothmer RV, Dayteg C, Rashal I, Tuvesson S and Weibull J 2004.Inter simple sequence repeat analysis of genetic diversity and relationships in cultivated barley of Nordic and Baltic origin. Hereditas **141**(2): 186–187.

Brockwell J, Bottomley PJ and Thies JE 1995. Manipulation of rhizobia microflora for improving legume productivity and soil fertility: a critical assessment. Plant Soil. **174**:143–180.

Burns RC and Hardy RWF 1975. Nitrogen fixation in bacteria and higher plants. New York, NY: Springer-Verlag.

Chen WM, Moulin L, Bontemps C, Vandamme P, Béna G and Boivin-Masson C 2003. Legumes symbiotic nitrogen fixation by beta-proteobacteria is widespread in nature. J. Bacteriol.**185**:7266-7272.

Compant S, Clément C and Sessitsch A 2010. Plant growth-promoting bacteria in the rhizo- and endosphere of plants: their role, colonization, mechanisms involved and prospects for utilization. Soil Biol. Biochem. **42**: 669-678.

Cooke RJ 1984. The characterization and identification of crop cultivars by electrophoresis. Electrophoresis **5**: 59-72.

Date RA 1970. Microbiological problems in the inoculation and nodulation of legumes.Plant and Soil.**32**: 703-725.

Dénarié J, Debellé F, Truchet G and Promé JC 1993.Rhizobium and legume nodulation: A molecular dialogue. *In*: Palacios R, Mora J and Newton WE (Eds) New horizons in nitrogen fixation. Kluwer, Dordrecht, pp 19-30.

Duenas M, Hernandez T and Estrella I 2002.Phenolic composition of the cotyledon and the seed coat of lentils (*Lens culinaris* L.).Eur Food Res Techno. **215**: 478-483.

Duenas M, Sun B, Hernandez T, Estrella I and Spranger MI 2003. Proanthocyanidin composition in the seed coat of lentils (*Lens culinaris* L.). J. Agric. Food Chem. **51**: 7999-8004.

Egamberdieva D, Li Li, Lindström K and Räsänen L 2016. A synergistic interaction between salt tolerant *Pseudomonas* and *Mezorhizobium* strains improves growth and symbiotic performance of liquorice (*Glycyrrhiza uralensis* Fish.) under salt stress. Appl. Microbiol. Biotech. **100** (6): 2829-2841.

Elias SM, Hossain MS, Sikder FS, Juber A and Karim MR 1986.Identification of constraints to pulse production with special reference to present ferming systems.Annual Report of the Agricultural Economic Division, BARI, Joydevpur.

Elsheikh EAE and Wood M 1990. Effect of salinity on growth, nodulation and nitrogen yield of chickpea (*Cicer arietinum* L.). J. Exp. Bot. **41**:1263-1269.

FAO 2008.FAO Land and Plant Nutrition Management Service.http://www.fao.org.

FAO 2009.High Level Expert Forum—How to Feed the World in 2050, Economic and Social Development, Food and Agricultural Organization of the United Nations, Rome, Italy.

Flowers TJ 2004.Improving crop salt tolerance.J. Exp. Bot.**55**(396): 307-319.

Freiberg C, Fellay R, Bairoch A, Broughton W J, Rosenthal A and Perret X 1997. Molecular basis of symbiosis between *Rhizobium* and legumes. Nature. **387**: 394-401.

Fride M and Middelboe V 1977.Measurement of amount of nitrogen fixed by legume crop. Plant and soil **47**: 713-715.

Gage DJ 2004.Infection and invasion of roots by symbiotic, nitrogen-fixing rhizobia during nodulation of temperate legumes.Microbiol Mol. Biol. Rev.**68**:280-300.

Geurts R and Bisseling T 2002. Rhizobium nod factor perception and signalling. Plant Cell **14**:S239-S249.

Ghassemi F, Jakeman AJ and Nix HA 1995. Salinisation of land and water resources: human causes, extent, management and case studies. UNSW Press: Sydney.

Graham PH and Parker CA 1964. Diagnostic features in the characterisation of the root-nodule bacteria of legumes. Plant Soil **20**: 383-6.

Greenway H and Munns R 1980.Mechanism of salt tolerance in nonhalophytes. Annu. Rev. Plant Physiol. **31**: 149-190.

Hafeez FY, Aslam Z and Malik KA 1988.Effect of salinity and inoculation on growth, nitrogen fixation and nutrient uptake of *Vigna radiata* (L.)Wilczek. Plant Soil **106**: 3-8.

Haque MZ and Saifuzzaman M 2003. Social and environmental effects of shrimp cultivation in Bangladesh: notes on study methods. *In*: Rahman M (ed). Globalization, Environmental Crisis and Social Change in Bangladesh, Dhaka, Bangladesh, UPL.

Hasegawa PM, Bressan RA, Zhu JK and Bohnert HJ 2000.Plant cellular and molecular responses to high salinity.Annu. Rev. Plant Physiol. Plant Mol. Biol.**51**: 463-499.

Hossain MZ, Rasel MIU and Samad R 2016a. Soil moisture effects on the growth of lentil (*Lens culinaris* Medik.) varieties in Bangladesh. Mol **16**: 30-40.

Hossain MZ, Hasan MM, Ferdous J and Hoque S 2016b.Growth responses of lentil (*Lens culinaris*Medik.) varieties to the properties of selected soils in Bangladesh. Mol. **16**: 18-29.

Hossain MZ, Hasan MM and Kashem MA 2018. Intervarietal variation in salt tolerance of lentil (*Lens culinaris* Medik.) in pot experiments. Bangladesh J. Bot. **47**(3): 405-412.

Islam MS 2003.Perspectives of the coastal and marine fisheries of the Bay of Bengal, Bangladesh. Ocean Coast. Manage.**46**: 763-796.

Ismet B and Fikret Y 2011.Characterization of bean (*Phaseolus vulgaris* L.) cultivars grown in Turkey by SDS–PAGE of seed proteins. Pak. J. Bot. **43**(2): 1085-1090.

James RA, Blake C, Byrt CS and Munns R 2011. Major genes for Na^+ exclusion, Nax1 and Nax2 (wheat HKT1; 4 and HKT1; 5), decrease Na^+ accumulation in bread wheat leaves under saline and waterlogged conditions. J. Exp. Bot. **62**(8): 2939–2947.

Jamil M, Lee CC, Rehman SU, Lee DB, Ashraf M and Rha ES 2005.Salinity (NaCl) tolerance of brassica species at germination and early seedling growth.Electr. J. Environ., Agric. Food Chem. **4**(4): 970-976.

Jimenez JS, Debouk DG and Lynch JP 2002. Salinity tolerance in *Phaseolus* species during early vegetative growth. Crop Sci. **42**: 2184-2192.

Juma KA and Averbeke V 2005.Response of Muxe to N and P availability in pots.*In*: Tenywa JS, Adipala E, Nampala P, Tussiime G, Okori P, Kyanmuhangire W (eds.). Afr. Crop Sci. Proceedings **7**: 1179-1182.

Kanawapee N, Sanitchon J, Srihaban P and Theerakulpisut P 2013. Physiological changes during development of rice (*Oryza sativa* L.) varieties differing salt tolerance under saline field condition. Plant Soil **370**: 89-101.

Kapoor K and Srivastava A 2010. Assessment of salinity tolerance of *Vingamungo* var. Pu-19 using ex vitro and in vitro methods.Asian J. Biotech.**2**(2): 73-85.

Katerji N, Hoorn VJW, Hamdy A and Mastrorelli M 2001. Salt tolerance of crops according to three classifications methods and examination of some hypothesis about salt tolerance. Agric. Wat. Manage. **47**: 1-8.

Katerji N, Hoorn VJW, Hamdy A, Mastrorilli M, Oweis T and Erskine W 2001. Response of two varieties of lentil to soil salinity. Agric. Wat. Manage. **47**: 179–190.

Krishnamurthy L, Serraj R, Hash CT, Dakheel AJ and Reddy BVS 2007.Screening sorghum genotypes for salinity tolerant biomass production.Euphytica**156**: 15-24.

Kurdali F, Kalifo K and Al Schamma M 1997.Cultivar differences in nitrogen assimilation, partitioning and mobilization in rain-fed grown lentil. Field Crops Res. **54**: 235-243.

Ladizinsky G 1979. The origin of lentil and its wild genepool. Euphytica **28**: 179-187.

Lindström K and Martínez-Romero E 2007. International committee on systematics of prokaryotes subcommittee on the taxonomy of Agrobacterium and Rhizobium: minutes of the meeting, 23-24 July 2006, Århus, Denmark. Int. J. Syst. Evol.Microbiol.**57**:1365-1366.

Long SR 2001. Gene and signals in the Rhizobium-legume symbiosis. Plant Physiol. **125**:69-72.

Lozupone CA and Knight R 2007. Global patterns in bacterial diversity. P. Natl. Acad. Sci. USA **104**: 11436-11440.

Lutts S, Majerus V and Kinet JM 1999. NaCl effects on proline metabolism in rice (*Oryza sativa*) seedlings. Physiol. Plant **105**: 450-458.

MacLean AM, Finan TM and Sadowsky MJ 2007. Genomes of the symbiotic nitrogen-fixing bacteria of legumes. PlantPhysiol. **144**:615-622.

MacVicar R, Garman WL and Wall R 1951. Studies on nitrogen fertilizer utilization using ^{15}N. Soil Sci. Soc. Am. Proc. **15**: 265-268.

Mahajan S and Tuteja N 2005. Cold, salinity and drought stresses: an overview. Arch. Biochem. Biophys. **444**(2): 139–158.

Marilley J and Aragno M 1999. Phylogenetic diversity of bacterial communities differing in degree of proximity of *Lolium perenne* and *Trifolium repens* roots. Appl. Soil Ecol.**13**:127-136.

Marsudi NDS, Glenn AR and Dilworth MJ 1999. Identification and characterization of fast- and slow-growing root nodule bacteria from south-western Australian soils able to nodulate *Acacia saligna*. Soil Biol. Biochem. **31**:1229–38.

McWilliam JR 1986.The national and international importance of drought and salinity effects on agricultural production. Aust. J. Plant Physiol. **13**:1-13.

Memon SA, Hou X and Wang LJ 2010. Morphological analysis of salt stress response of pak Choi. EJEAFChe **9**(1): 248-254.

Miah MY, Mannan MA, Quddus KG, Mahmud MAM and Badia T 2004.Salinity on cultivable land and its effects on crops. Pakistan J. Biol. Sci. **7**(8): 1322-132.

Micheni A, Kihanda F and Irungu J 2004.Soil organic matter (SOM).The basis for improved crop production in arid and semi arid climate of Eastern Kenya. *In*: Managing nutrient cycles to sustain soil fertility in Sub-Saharan Africa, Bationo A (ed.). pp. 608.

Misra N and Gupta AK 2005.Effect of salt stress on proline metabolism in two high yielding genotypes of green gram. Plant Sci. **169**(2): 331-339.

Mohamed SH, Smouni A and Neyra M 2000. Phenotypic characteristics of root-nodulating bacteria isolated from *Acacia* grown in Libya. Plant Soil. **224**: 171–83.

Mugwe J, Mugendi D, Okoba B, Tuwei P and O'Neill M 2004. Soil conservation and fertility improvement using leguminous Shrubs in central highlands of Kenya: NARFP Case study. *In*: Managing nutrient cycles to sustain soil fertility in sub-Saharan Africa, Bationo A (ed.). pp. 608.

Munns R 2005. Genes and salt tolerance: bringing them together. New Phytol. **167**(3): 645-663.

Munns R and Tester M 2008. Mechanisms of salinity tolerance. Annu. Rev. Plant Biol. **59**: 651-681.

Murphy RW, Sites JW, Buth DG and Haufler CH 1990. Protein and isozyme electrophoresis.*In*: Hillis DH and Moritz C (Eds.), Molecular Systematics. Sinauer Assoc., Sunderland, MA, pp. 45-126.

Nedjimi B, Daoud Y and Touati M 2006. Growth, water relations, proline and ion content of invitro cultured *Atriplexhalimus* sub sp. *Schweinfurthii* as affected by $CaCl_2$. Commun. Biometry Crop Sci. **1**(2): 79-89.

Netondo GW, Onyango JC and Beck E 2004.Response of growth, water relations, and ion accumulation to NaCl salinity. Crop Sci.**44**: 797-805.

Orak A and Ates E 2005.Resistance of salinity stress and available water levels at the seedling stage of the common vetch (*Vicia sativa* L.). Plant Soil Environ. **51**(2): 51-56.

Pedalino M, D-Urzo MP, Donnc GD, Grillo S and Rao R 1992.The structure of Cowpea (*Vigna unguiculata* L. Walp) seed storage proteins. Seed Sci. Technol. **2**: 223-231.

Peoples MB, Herridge DF and Ladha JK 1995. Biological nitrogen fixation: an efficient source of nitrogen for sustainable agricultural production. Plant Soil. **174**: 3-28.

Peoples MB, Ladha JK and Herridge DF 1995. Enhancing legume N_2 fixation through plant and soil management.Plant Soil.**174**:83–101.

Perret X, Staehelin C and Broughton WJ 2000. Molecular basis of symbiotic promiscuity.Microbiol Mol. Biol. Rev.**64**:180-201.

Pessarakli M, Huber JT and Tucker TC 1989. Protein synthesis in grean beans under salt stress with two nitrogen sources. J. Plant Nut. **12**: 1261–1377

Petersen L and Shireen S 2001.Soil and water salinity in the coastal area of Bangladesh. Soil Research Development Institute, Dhaka.

Pohili RM and Raven PH 1981. Advances in Legume Systematics. Royal Botanic Garden, KEW.

Przbylska J 1986. Identification and classification of the *Pisum* genetic resources with the use of electrophoretic protein analysis. Seed Sci. Technol. **111**: 529-543.

Purseglove JW 1968. Tropical Crops Dicotyledons 1. Longmann, Green and Co. Ltd. pp. 279.

Rahnama A, James RA, Poustini K and Munns R 2010.Stomatal conductance as a screen for osmotic stress tolerance in durum wheat growing in saline soil.Func. Plant Biol. **37**(3): 255–263.

Rai R 1992. Effect of nitrogen levels and Rhizobium strains on symbiotic N fixation and grainyield of Phaseolus vulgaris L.genotypes in normal and saline-sodic soils. Biol. Fertil. Soils **14**: 293-299.

Reddy M, Sarla N and Siddiq EA 2002. Inter simple sequence repeat (ISSR) polymorphism and its application in plant breeding. Euphytica **128**: 9-17.

Rengasamy P 2006.World salinization with emphasison Australia. J. Exp. Bot. **57**: 1017-1023.

Rozema J and Flowers T 2008. Ecology: crops for a salinized world. Science **322**:1478–1480.

Sanchez PA, Sheperd KD, Soule MG, Place FM, Buresh RJ, Nanne-Marie Nl, Mokwunje AU, Kwesiga FR, Ndiritu CG and Woomer PL 1997. Soil fertility replenishment: an investment in natural resource capital. *In*: Replenishing soil fertility in Africa, Buresh RJ, Sanchez PA, Calhoun F, Soil Sci. Soc. Am. Special Publi. No. 51.

Sandhu JS and Singh S 2007. History and origin, *In*: Lentil an ancient crop for modern times. Yadav SS, McNeil DL and Stevenson PC, Springer, Dordercht, the Netherlands. pp 1-11.

Sauer D and Burroughs R 1986. Disinfection of seed surfaces with sodium hypochlorite. Phytopathology **76**(7): 745-749.

Schultze M and Kondorosi A 1998. Regulation of symbiotic root nodule development.Annu. Rev. Gene. **32**:33-57.

Sellstedt A, Staahl L, Mattsson M, Jonsson K and Hoegberg P 1993. Can the ^{15}N dilution technique be used to study N_2 fixation in tropical tree symbioses as affected by water deficit? J. Exp. Bot.**44**: 1749-1755.

Senanayake L, Knievel DP and Stevena SE 1987.Nodulation and symbiotic nitrogen fixation of cowpea (*Vigna ungiculata* L). Plant Soil **99**: 435-439.

Serraj R 2002. Response of symbiotic nitrogen fixation to drought and salinity stresses. Physiol. Mol. Biol. Plants **8**(1): 77–86.

Shannon, Michael C, Grieve CM and Francois Le 1994. Whole-plant response to salinity. Plant environment interaction. pp. 199-244.

Sharma SK and Mukhopaday S 1963. Karyotype consistency in different strains of *Lens esculenta* Moench. as worked out through recent techniques. Indian Agri. **7**: 103-111.

Siler B, Misic D, Filipovic B, Popovic Z, Cvetic T and Mijovic A 2007. Effects of salinity on in vitro growth and photosynthesis of common centaury (*Centaurium erythraea* Rafn.). Arch. Biol. Sci. **59**(2): 129-134.

Singh J and Singh JP 1995.Land degradation and economic sustainability. Ecol. Econ. **15**(1): 77-86.

Singleton PW and Bohlool BB 1984.Effect of salinity on nodule formation by soybean. Plant Physiol. **74**: 72-76.

Smalla K, Wieland G, Buchner A, Zock A, Parzy J, Kaiser S, Roskot A, Heuer H and Berg G 2001. Bulk and rhizosphere soil bacterial communities studied by denaturing gradient gel electrophoresis: plant-dependent enrichment and seasonal shifts revealed. Appl. Environ. Microbiol.**67**(10): 4742-4751.

Spaink HP 2000. Root nodulation and infection factors produced by rhizobial bacteria. Annu. Rev. Microbiol. **54**:257-288.

SRDI 2010.Soil salinity in Bangladesh 2000. Soil Resource Development Institute (SRDI), Ministry of Agriculture, Government of the People's Republic of Bangladesh, Dhaka, Bangladesh.

Sultana T and Ghafoor A 2008. Genetic diversity in *ex-situ* conserved *Lens culinaris* for botanical descriptors, biochemical and molecular markers and identification of landraces from Indigenous Genetic Resources of Pakistan. J. Integr. Plant Biol. **50**: 484-490.

Taiz L and Zeiger E 2006.Plant Physiology, Ed 4.Sinauer Associates, Sunderland, MA.

Tanji KK 1990. Nature and extend of agricultural salinity. Agricultural salinity assessment and management, Tanji KK (ed), Published by American Society of Civil Engineers, pp.1-18.

Tanwir F, Saboor A and Newaz N 2003. Soil salinity and the livelihood strategies of small farmers: a case study in Faisalabad District, Punjab, Pakistan. Int. J. Agric. Biol. **5**(4): 440-441.

Tate RL 1995. Soil microbiology (symbiotic nitrogen fixation) New York, N.Y: John Wiley & Sons, Inc. pp. 307-333.

Tort N and Turkyilmaz B 2004. A physiological investigation on the mechanisms of salinity tolerance in some barley culture forms. J. Agri. Faculty of Ege Univ. **27**: 1-16.

Turhan E and Eris A 2005. Changes of micronutrients, dry weight, and chlorophyll contents in strawberry plants undersalt stress conditions. Commun. Soil Sci. Plant Anal. **36**: 1021-1028.

van Hoorn JW, Katerji N, Hamdy A and Mastrorilli M 2001.Effect of salinity on yield and nitrogen uptake of four grain legumes and on biological nitrogen contribution from the soil. Agr. Water Manag. **51**: 87–98.

Wang N, Hatcher DW, Toews R and Gawalko EJ 2009. Influence of cooking and dehulling on nutritional composition of several varieties of lentils (*Lens culinaris*). LWT -Food Science and Technology **42**: 842-848.

Yang H, Hu J, Long X, Liu Z and Rengel Z 2016. Salinity altered root distribution and increased diversity of bacterial communities in the rhizosphere soil of Jerusalem artichoke. Sci. Rep. **6**: 20687.

Yates RJ, Howieson JG, Nandasana KG and O'Hara GW 2004.Root-nodule bacteria from indigenous legumes in the north-west of Western Australia and their interaction with exotic legumes. Soil Biol. Biochem. **36**:1319–29.

Yeo AR 1983. Salinity resistance: Physiologies and prices. Physiol. Plant **58**: 214-222.

Yoseph B, Botha AM and Myburg AA 2005. A comparative study of molecular and morphological methods of describing genetic relationships in traditional Ethiopian highland maize. Afr. J. Biotechnol. **4**(7): 586–595.

Yurtseven E, Kesmez GD and Unlukara A 2005. The effects of water salinity and potassium levels on yield, fruit quality and water consumption of a native central Anatolian tomato species (*Lycopersicon esculantum*). Agric. Water Manag. **78**: 128-135.

Yuzbasßioglu E, Acik L and Ozcan S 2008.Seed protein diversity among lentil cultivars. Biol. Plantarum **52**:126–128.

Zahran HH 1999. *Rhijobium*-Legume Symbiosis and Nitrogen Fixaion under Severe Conditions and in an Arid Climate.Microbiol. Mol. Biol. Rev. **63(**4): 968-989.

Zahran HH, Ahmed MS and Afkar EA 1995.Isolation and characterization of nitrogen-fixing moderate halophilic bacteria from saline soils of Egypt. J. Basic Microbiol**. 35**: 269-275.

Zapata F, Danso SKA, Hardarson G and Fried M 1987. Nitrogen fixation and translocation in field grown fababean. Agron. J. **79**: 505-509.

APPENDIX

Appendix 1. Mean values with standard error mean of the effects of salt concentration on chlorophyll and root-shoot parameter.

Conc. (mM)	Shoot height (cm)	Root height (cm)	Chlorophyll	Shoot fresh wt (g)	Shoot dry wt (g)	Root fresh wt (g)	Root dry wt (g)	Nodule per plant
0	14.30±0.99	9.59±0.15	17.27±3.87	0.25±0.04	0.08±0.02	0.10±0.006	0.01±0.00	1.00±0.08
50	11.63±0.79	9.31±0.88	16.81±3.59	0.29±0.02	0.08±0.003	0.09±0.009	0.01±0.00	1.07±0.11
100	11.45±1.37	9.00±0.56	11.87±3.76	0.33±0.003	0.11±0.009	0.10±0.01	0.01±0.00	1.30±0.01
200	11.85±0.59	8.27±0.41	11.53±1.67	0.25±0.02	0.09±0.007	0.09±0.01	0.009±0.00	0.79±0.03
F-ratio	1.86	1.01	0.85	2.37	1.97	0.46	1.00	9.85
P value	0.21	0.44	0.50	0.15	0.19	0.72	0.44	0.005

Appendix 2. Accumulation of proline in leaves of lentil under different salt concentrations.

Concentration (mM)	Proline (µg/gm)
0	0.042±0.011
50	0.133±0.033
100	0.188±0.039
200	0.210±0.019
F-ratio	7.26
P value	0.01

Appendix 3. The number of the different bacterial colonies (Small colony, medium colony and large colony) obtained from root nodule at different concentrations of salt (0 mM, 50 mM, 100 mM and 200 mM) after 24 hours of incubation.

Nodule ID	Concentration	Replicate	small	Medium	Large
0N1	0	1	405	0	0
0N2	0	2	173.77	0	0
0N3	0	3	56.67	0	0
50N1	50	1	560	2.67	4
50N2	50	2	483.33	1.33	2.67
50N3	50	3	221.67	10.33	30
100N1	100	1	548.33	11.67	17.67
100N2	100	2	510	13	19
100N3	100	3	416.67	17	34.67
200N1	200	1	330	31.33	30.67
200N2	200	2	436.67	7.33	29.33
200N3	200	3	406.67	14.67	28.33

Appendix 4. Mean values with standard error mean of different colonies (Small colony, medium colony and large colony) from nodule at different concentrations of salt (0 mM, 50 mM, 100 mM and 200 mM) after 24 hours.

Concentration	Small	Medium	Large
0	211.81±102.34	0.00±0.00	0.00±0.00
50	421.67±102.42	4.78±2.80	12.22±8.89
100	491.67±39.1	13.89±1.60	23.78±5.46
200	391.11±55.01	17.78±7.10	29.44±0.68
F-ratio	2.42	4.38	6.23
P value	0.14	0.04	0.02

Appendix 5. The number of different bacteral colonies (Small colony, medium colony and large colony) obtained from rhizosphere soil at different concentrations of salt (0 mM, 50 mM, 100 mM and 200 mM) after 24 hours of incubation.

Soil ID	Soil Type	Concentration(mM)	Replicate	Small	Medium	Large
0R1	Rhizosphere	0	1	148.25	12.5	16.25
0R2	Rhizosphere	0	2	38	0	0.5
0R3	Rhizosphere	0	3	50	0	0.25
50R1	Rhizosphere	50	1	339	16.25	19.5
50R2	Rhizosphere	50	2	77.5	1.5	3.25
50R3	Rhizosphere	50	3	70	0	0.75
100R1	Rhizosphere	100	1	381.25	9.75	11.5
100R2	Rhizosphere	100	2	61.25	1	1.5
100R3	Rhizosphere	100	3	197.5	0	0
200R1	Rhizosphere	200	1	216.25	13.5	13.5
200R2	Rhizosphere	200	2	332.5	3.5	4.5
200R3	Rhizosphere	200	3	83.75	0	0.25

Appendix 6. The number of different bacterial colonies (Small colony, medium colony and large colony) obtained from bulk soil at different concentrations of salt (0 mM, 50 mM, 100 mM and 200 mM) after 24 hours of incubation.

Soil ID	Soil type	Concentrations (mM)	Replicate	Small	Medium	Large
0B1	Bulk	0	1	69.25	0.25	0
0B2	Bulk	0	2	157.5	0.25	2.5
0B3	Bulk	0	3	303.75	0	0
50B1	Bulk	50	1	65.5	14.5	0
50B2	Bulk	50	2	161.25	0	0
50B3	Bulk	50	3	127.5	0	0
100B1	Bulk	100	1	150	0	0.75
100B2	Bulk	100	2	36.25	0.75	1.25
100B3	Bulk	100	3	347.5	0	0
200B1	Bulk	200	1	190	0.5	0.25
200B2	Bulk	200	2	132.5	0	0
200B3	Bulk	200	3	218.75	0	0

Appendix 7. DNA sequence of *Rhizobium* isolates from root nodules by amplifying 16s rRNA gene following PCR.

Sample id	Sequence	Organisms name
R5F	GGTGGCATGGCATGGGCGCAGCCTGATCCAGCCATGCCGCGTGAGTGAT GAAGGCCCTAGGGTTGTAAAGTTTAATTGGTTTAGATAATGACGGTATCC GGAGAAGAAGCCAAACTAACTTCGGGCCAGCAGCCGCGGTAATACGAA GGGGGCTAGCGTTGTTCGGAATTACTGGGCGTAAAGCGCACTTCCGCGG ATCGATCAGTCAGGGGTGAAATCCCAGGGCTCAACCCTGGAACTGCCTT TGATACTGTCGATCTGGAGTATGGAAGAGGTGAGTGGAATTCCGAGTGT AGAGGTGAAATTCGTAGATATTCGGAGGAACACCAGTGGCGAAGGCGG CTCACTGGTCCATTACTGACGCTGAGGTGCGAAAGCGTGGGGAGCAAAC AGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAATGTTAGCCG TCGGGCAGTATACTGTTCGGTGGCGCAGCTAACGCATTAAACATTCCGCC TGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGGCCC CGCACAAGATGAA	*Rhizobium*
R5R	CACGCATACTTGCGACGTACTCCCCAGGCGGATGTTTATGCGTTAGCTGC GCCACCGAACAGTATACTGATGGCGCTACTTTCATCGTTTACGGGGTGGA CTACAGGGAAGCGCTTCTTTCGTCGCTCCCAATTTTTCGCACCTCAACGT CAGTAATGGACCAGTGAGCCGCCTTCGCCACTGTTGACAAGTAAGGGGT CGGGGAAATTTCACCTCTACACTCGGAATTCCACTCACCTCTTCCAAACT CCAGATCGACAGTATCAAAGGGAGTTCCAGGGGTGAGCCCTGGGATTTC ACCCCCTGAATGATCGATCCGCCAAGGTGCGCTTTACGCCCAGTAATTCC GAACAACGGTAGCCCCCTTCGTATTAACGCGGCTGGTGGCACGAAGTTA GCCGGGTGTTTCTTCTCCGGATACCGTCATTATCTTCTCCGGTGAAAGAG CTTTACTATCCTAGGGGCTTCATCACTCACGCGGCATGATGGCATCATGC TTGCGCCCATTGTCATTATTCGCGATGCTGCCTCCAGTAGAATCTGGAAT GTGCTTCCGTAATATTATGTGTATCTCCCCCCCCCCCTCGGGGGGGGAGG GGGAAAAA	*Rhizobium*
R6F	CTCCTCGAGTTTACCGGCTTGCATATGACTGACGGAAAAGTCAAATAAG GGGCGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGTTCGGAATTACT GGGCGTAAAGCGCACGTAGGCGGATCGATCAGTCAGGGGTGAAATCCCA GGGCTCAACCCTGGAACTGCCTTTGATACTGTCGATCTGGAGTATGGAA GAGGTGAGTGGAATTCCGAGTGTAGAGGTGAAATTCGTAGATATTCGGA GGAACACCAGTGGCGAAGGCGGCTCACTGGTCCATTACTGACGCTGAGG TGCGAAAGCGTGGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACG CCGTAAACGATGATGTTAGCCGTCGGGCAGTATCTGTTCGGTGGCGCAG CTACGCTTAACTTCCGCTGGGGATACGGTCGCAGAATAAACCCAAAGAA TTGCGGGGCCGCCCAGAA	*Rhizobium*
R6R	CATCCCCAATAATCAAAAGGTTCTCGTTTTGGGGGCACGGCAACAGTCCT GTTTGCTCGCCACGCTTTCGCACCTCAGCGTCAGTAATGGACCAGTGAGC CGCCTTCGCCACTGGAAAAAGCTGGTGTCTACGAATTTCACCTCTACACT CGGAATTCCACTCACCTCTTCCATACTCCAGATCGACAGTATCAAAGGCA GTTCCAGGGTTGAGCCCTGGGATTTCTCCCCCGCCTGATCGATCCGCCAC GTGCGCTTTACGCCCAGTAATCCGAACAACGCTAGCCCCCTTCGTATTAC	*Rhizobium*

	CGCGGCTGCTGGCCCAAGTTAGCTCGTTTTTCCTTTCCGCACACCGTCTT ATCTTCTCCGGTGAAAGAGCTTTTCTACCTTAGGGCTTCATCACTCCGCG GATGGCTGGATCAGGCTTGCCCCTTTGCTTTTATCGCCACTGGTGCTCCC GTAGAATTCGGAA	
C 8 F	CCCTCCAGGACACCGTTAAAGAATGACGGACCGGAAAAATCACGTAATT CGGGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGTTCGGAATT ACTGGGCGTAAAGCGCTCGTCCCGCGGATCGATCAGTCAGGGGTGAAAT CCCAGGGCTCAACCCTGGAACTGCCTTTGATACTGTCGATCTGGAGTATG GAAGAGGTGAGTGGAATTCCGAGTGTAGAGGTGAAATTCGTAGATATTC GGAGGAACACCAGTGGCGAAGGCGGCTCACTGGTCCATTACTGACGCTG AGGTGCGAAAGCGTGGGGGAGCAAACAGGATTAGATACCCTGGTAGTCC ACGCCCGTAAAACGATGAATGTTAGCCGTCGGGCAGTATACTGTTCGGT GGCGCAGCCTAACCGCATTTAACATTCCCGCCTGGGGAGTACGGTCGCA AGATTAAAATCCAAAGGAATTTGACGGGGGGCCCGGCACAAGGA	*R* *hi* *zo* *bi* *u* *m*
C 8 R	CCGTAATACCTTGCGACGTACTCCCCAGGCGGATGTTTAATGCGTTAGCT GCGCCACCGAACAGTATACTGGAGGGTCTGCTTCATCGTTTACGGCGTG GACTACAGGGCAGTGCCTTTTGCTCCCCACGCTTTCGCACCTCAGCGTCA GTAATGGACCAGTGAGCCGCCTTCGCCACTGGTGACAAAGGAATAGGAG AGAGATTTCACCTCTACACTCGGAATTCCACTCACCTCTTCCATACTCCA GATCGCGACAGTATCAAAGGCAGTTCCAGGGTTGAGCCCTGGGATTTCA CCCCCCCACTGATCGATCCGCCTACGTGCGCTTTACGCCCAGTAATTCCG AACAACGCTAGCCCCCTTCGTATTACCGCGGCTGCTGGCACGAAGTTAG CCGGGGGTTCTTTCCGGAACCGCCTTTTTTTTTCTCGGGGAAAAGAAAGA GCTTTACAACCCTAGGGCCCGTATCACTCACGCGGGCATGGCTGGATCA GGGGTGGGCCCCTTGATATTTTCTTGGGGGGGGGGGTGCCTCCGAAAAA ATTGGG	*R* *hi* *zo* *bi* *u* *m*
C 9 F	CGCTGCCTGGCATGGGCGCAGCCTGATCCAGCCATGCCGCGTGAGTGAT GAAGGCCCTAGGGTTGTAAAGATATTCTCGAAGATAATGACGGTATCCG GAGAAGAAGAAAGCTAGCTTCGTGCCAGCAGCCGCGGTAATACGAAGG GGGCTAGCGTTGTTCGGAATTACTGGGCGTAAAGCGCTCGCAGGCGGAT CGATCAGTCAGGGGTGAAATCCCAGGGCTCAACCCTGGAACTGCCTTTG ATACTGTCGATCTGGAGTATGGAAGAGGTGAGTGGAATTCCGAGTGTAG AGGTGAAATTCGTAGATATTCGGAGGAACACCAGTGGCGAAAGCGGCTC ACTGGTCCATTACTGACGCTGAGGTGCGAAAGCGTGGGGGAGCAAACAG GATTAGATAACCCTGGTAGTCCACGCCCGTAAACGATGAATGGTTATCC GTCGGGCAGTATACTGTTTCGGTGGCGCAGCTAACGCATTAAAAATCCC GCCTGGGGGAGTACGGTCGCAAGATTAAAACTCCAAGGAATTGACGGGG GGCCCCCCCAAGAA	*R* *hi* *zo* *bi* *u* *m*
C 9 R	CTGAAAACCGGTGCCGACCGCTACTCCCCAGGCGGGGTGTTGTAATGCG TTACCTGCGCCACCGGAACAGTATACTGGTGGGCGTGGCATTCATCGTTT ACGGGTGGACTACAGGTAGTGTCCTTTTGCTCCCACGCTTCGCACCTCAG CGTCAGTAATGGACCAGTGAGCCGCCTTCGCCACTGGGGGAAAAGAAGG TCAGGGAATTTCACCTCTACACTCGGAATTCCCTCCCTCTTCGTACTCCA GATCGACAGTATCAAAGGCAGTTCCAGGGTTGAGCCCTGGGATTTCACC CCTGACTGATCGATCCGCCTACGTGCGCTTTACGCCCAGTAATTCCGGCA ACGCTAACCCCCTTCGTATTACCGCGGTGCTGGGACGAATTAGCGGGGG TTTTCTCCGTATCGCTTTCTCCCGGGAAGGGGCTACCTCCGGGCCTCACC TCCGGGGTGGTGGGGGGGGTGCGCCCCGCGGGAGGCACAGAACCACAA AGAATCGGATCGTTTGGGCGCCCCCTCTTAAATCTGGGGGGGGGGGGGG GAGACAACAAAAACAATAATGCTACATCCAAACGG	*R* *hi* *zo* *bi* *u* *m*