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Ph.D. in Biology

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REPUBLIC OF TURKEY
GAZIANTEP UNIVERSITY
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CHARACTERIZATION OF CHICKPEA NODULE BACTERIA IN TURKEY

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in

Biology

Gaziantep University

Supervisor

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Ahmed MAHMOUD

ABSTRACT

CHARACTERIZATION OF CHICKPEA NODULE BACTERIA IN TURKEY

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Chickpea (*Cicer arietinum* L.) is among the first cultivated pulses in Turkey. Since Turkey soils are deficient in nitrogen, rhizobia can replace chemical fertilizers to increase chickpea yield at low cost. Also, nodules harbor non-nodulating endophytes (NEB) that can promote the plant growth. In this study, we aimed to investigate the diversity of RNR and NEB bacteria inside chickpea nodules. A total of 120 RNR and 13 NEB isolates, from 17 cities in 6 provinces in Turkey, were characterized on the basis of morphological, physiological, and biochemical traits. Besides, the NEB isolates were further identified on molecular basis using REP-PCR and 16S rRNA. Ninty percent of the RNR isolates displayed mucously, circular, smooth-margined colonies. Also, 11 out of 13 non-nodulating isolates displayed light pink colonies. All isolates in both groups endured up to 2% NaCl, grew optimally at 25-35°C and at pH 6-8. They all were gram-negative, fast growers and catalase and oxidase positive, while negative for amylase and methyl red. More than 95% of isolates in both groups utilized different compounds as sole carbon and nitrogen sources. The majority of isolates in both groups were insensitive to the heavy metals Zn, Cu and Cr, and resistant to the antibiotics such as kanamycin, streptomycin and tetracycline. Numerical analysis separated the isolates in each group into three clusters. Clusters 1, 2 and 3 of the RNR group came with 113, 5 and 2 isolates, respectively, and were respectively close to *Mesorhizobium ciceri, M. mediterraneum* and *Mesorhizobium* sp. Eight representative isolates of NEB group were identified via 16S rRNA sequencing with 99% similarity to 4 genera: *Rahnella, Enterobacter, Pseudomonas, and Rhizobium*. This showed that NEB inside nodules were as diverse as RNR bacteria.

Key Words: Rhizobia, Chickpea, Phenotypic Characterization, Diversity

ÖZET

TÜRKİYE'DE NOHUT NÖDUL BACTERIA'NIN KARAKTERİZASYONU

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Nohut (*Cicer arietinum* L), Türkiye'de kültüre alınan ilk tane baklagillerden biridir. Türkiye'deki topraklarda azot eksikliği vardır, rhizobia nohutta düşük girdi ile verimi artırarak kimyasal gübrelerin yerini alabilir. Ayrıca, nodüller bitki gelişimini teşvik eden nodüle etmeyen endofitleri (NEB) bulundurur. Bu çalışmada, nohut nodüllerindeki rhizobial (RNR) ve NEB bakterilerinin çeşitliliğinin belirlenmesi amaçlanmıştır. Türkiye'de 6 bölge ve 17 ilden toplamda 120 RNR ve 13 NBR izolat, morfolojik, fizyolojik ve biyokimyasal özellikler ile karakterize edilmiştir. NEB izolatları moleküler anlamda REP-PCR ve 16S rRNA analizleri ile tanımlanmıştır. RNR izolatları %90 oranda mukoz, dairesel ve düz kenarlı koloniler oluşturmuştur. Ayrıca 13 rizobial olmaya izolatın 11 tanesi açık pembe renkli koloni gelişimi sergilemiştir. Her iki grubun tüm izolatları %2 NaCI dayanıklık göstermiş, optimal gelişme 25-35°C'de ve pH 6-8'de olmuştur. Tamamı gram-negatif, hızlı gelişen, katalaz ve oksidaz pozitif iken amilaz ve metil kırmızısı negatif olarak saptanmıştır. Her iki gruba giren izolatların %95'i karbon ve azot kaynağı olarak farklı bileşikleri kullanmıştır. Her iki gruptaki izolatların büyük bir bölümü Zn, Cu ve Cr ağır metallerine hassasiyet göstermemiş, kanamisin, streptomisin ve tetrasikline dayanıklılık sergilemiştir. Numerik analizler her gruptaki izolatları 3 kümeye ayırmıştır. RNR grubunun 1, 2 ve 3. kümeleri sırası ile 113, 5 ve 2 izolat ile temsil edilmiş, bu izolatlar sırası ile *Mesorhizobium ciceri*, *M. mediterraneum* ve *Mesorhizobium* sp. benzerliği sergilemiştir. NEB grubuna dahil olan 8 temsili izolat, 16S rRNA sekansı ile tanımlanmış, *Rahnella*, *Enterobacter*, *Pseudomonas* ve *Rhizobium* olmak üzere 4 cinse %99 oranında benzerlik göstermiştir.

Anahtar kelimeler: Rhizobia, Endofit, Nohut, Fenotipik Karakterizasyon, Çeşitlilik

To my beloved Family

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LIST OF ABBREVIATIONS

Amp	Ampicillin
BCP	Bromocresol purple
BLAST	Basic Local Alignment Search Tool
BNF	Biological nitrogen fixation
BTB	Bromothymol blue
CR	Cong red
CSP	Cold shock protein
EBI	European Bioinformatics Institute
EMBL	European Molecular Biology Laboratory
Ery	Erythromycin
FAO	Food and Agriculture Organization
gDNA	Genomic DNA
GPA	Glucose Peptone Agar
GPS	Global positioning system
HSP	Heat shock proteins
IAA	Indole acetic acid
KA	Kanamycin
MR	Methyl red
NA	Nalidixic acid
NCBI	National Center for Biotechnology Information

NEB	Nodule endophytic bacteria
PCR	Polymerase chain reaction based techniques
REP	Repetitive Extragenic Palindromic
RFLP	Restriction fragment length polymorphism
RNB	root-nodule bacteria
RNR	Root-nodulating rhizobia
SPSS	Statistical Package for the Social Science
Str	Streptomycin
TAE	Tris-Acetic Acid EDTA
TE	Tris-EDTA
Tetr	Tetracycline
Trypt	Tryptophan
YMA	Yeast extract mannitol agar

CHAPTER I

INTRODUCTION

1.1 Background

Despite its plenitude in the atmosphere, nitrogen is found in an innert form (N_2) which is only directly accessible to certain bacteria and archaea capable of expressing the nitrogenase enzyme which “fixes” atmospheric nitrogen into ammonia at high energetic costs. The ongoing consumption of the nitrogen resources from soil and the need for excessive crop production have resulted in a growing emphasis on finding alternatives to protect the restricted and finite resources of the nitrogen element in soil. Because only a small percentage of the entire agricultural demand for nitrogen is satisfied via organic and man-made fertilizers, the other part must be fulfilled through the soil pool and via natural processes such as biological fixations (Sprent, 2007).

Nitrogen is provided to cropping systems as industrial fertilizers to promote production. The industry of synthetic fertilizers depends on the natural gas and hence this links the fertilizer prices (and in turn to crop prices) to the natural gas prices. Synthetic nitrogen fertilizers are unavailable to much of the developing world and cause ecological and human health problems. Moreover, about 40-60% of the nitrogen applied to soil as urea is wasted via volatilization, drain-off, denitrifying microorganisms and by leaching. Leached nitrates cause diseases susceptibility, extensive growth which results in lodging of crops and reduces quality of seeds. Ongoing application of synthetic fertilizers might lead to metal contaminations and lowers vitamin C and carotene ingredients in vegetables and fruits. Meanwhile, synthetic fertilizers result in malnutrition because of the degradation of proteins and sugars. Hence, this leads to increased production costs and decreased food security. It also threats the lives and livelihood of human beings. Therefore, there is a growing interest in sustainable and secure food production (Yasin, 2007). Atmospheric elemental dinitrogen is fixed into compound nitrogen by diazotrophic bacteria. Thanks to symbiosis between leguminous plants and root-nodule diazotrophic

bacteria like *Rhizobium* and *Bradyrhizobium*, atmospheric dinitrogen is fixed in the root nodules via the process of biological nitrogen fixation (BNF) due to the action of the nitrogenase enzyme. Undoubtedly, BNF receives a universal interest as it renders lesser reliance on costly chemical nitrogen fertilizers for promoting yields of legume plants. The importance of symbioses between rhizobia and legumes is not negligible and imaginable for a plant living in a soil with nitrogen deficiency where the nitrogen fixing bacteria can supply the plant with the desired amount of nitrogen whenever the plant needs this with no need to fertilizers. BNF occurs effectively and expeditiously during the symbiosis (Sprent, 2007). Being soil-borne bacteria having the capability to infect root hairs of legumes, rhizobia can initiate nitrogen fixing nodules where the atmospheric nitrogen is fixed into ammonia. Also, the symbiosis between legumes and their microsymbiont species of rhizobia is very specific (Mishra et al., 2012). Recently, the evaluation of diversity of rhizobia populations in many geographical regions worldwide has received increasing attentions (De Meyer et al., 2015). BNF furnishes the cropland with about sixteen percent of the whole nitrogen input and is considered the potential to reduce the manufactured N-fertilizers. BNF is thus the cheapest and the best environmentally friendly procedure for alleviating environmental pollution and the deterioration of nature (Ramaekers et al., 2013). The nitrogen fixed via BNF is mostly used for the plant growth and development. This accounts to about 60% of the global nitrogen budget and is eco-friendly and cost effective. The symbiotic association that forms BNF is affected by various factors such as the rhizobia strains, host legumes and biotic and abiotic soil factors.

Leguminous plants are considered of vital importance in the nutrition of both humans and animals and are cultivated universally under a large scale of environmental conditions as pioneer crops and as sources of the assimilated nitrogen (Nyfeler et al., 2011). The symbiotic system in which legumes and rhizobia cooperate for biological nitrogen fixation is considered the most efficient one (Egamberdieva et al., 2017). Because they are able to grow in N- deficient soils, legume plants act as frontiers for soil stability and fertility and they protect soil against desertification and erosion. Native legumes are expected sources of various indigenous populations of rhizobia and nodules are expected to contain a mixture of rhizobia and other endophytic bacteria (Moulin et al., 2004). The significance of legumes is agriculturally

undeniable and thus natural populations of both rhizobia and other associative endophytes are being intensely characterized for their diversity in different cultivated and wild legumes (Naz et al., 2009).

Like most legumes, chickpea performs BNF and increases the combined N input to soil. Moreover, more than seventy percent of the chickpea nitrogen demands is fulfilled through symbiosis with nodulating bacteria, rendering chickpea symbiosis a great agricultural interest. Moreover, using selected rhizobia inoculants on chickpea has shown positive impacts on its outputs (Gundi et al., 2018). In Turkey, more than sixteen percent of protein consumption is provided by chickpea. In 1984, Turkey produced more than one-third of the world's chickpea. However, Turkey's production of chickpea is in decline due to both economical and phytopathological problems (Aybegün et al., 2014).

Inside root nodules of legumes, there are also various non-nodulating bacteria, which affect the existence, nodulation and output of the crop (Tariq et al., 2014). Non-nodulating bacteria exist inside the nodule tissues with high densities without rendering observable harming or gaining profits other than shelter (Kobayashi and Palumbo, 2000). These endophytes are somehow protected from the competition and stresses in the soil environment and can enhance growth of the macrosymbiont via excreting plant growth promoting substances (Patel et al., 2012). There has been no evidence that such bacteria can trigger nodule formation, but these non-nodulating bacteria may get access to the nodule through the connection between nodule and the root. Zgadzaj et al. (2015) reported that endophytic bacteria might also infiltrate nodules via the infection thread induced by rhizobia.

Characterization of the diversity among the rhizobia strains and other nodule endophytic bacteria is necessary for getting benefit of the genetic resources to improve BNF. To identify root nodule rhizobia and other associative NEB bacteria, many methods have been employed including phenotypic, biochemical and molecular approaches. The development of molecular genetic assays has greatly accelerated such investigations (Naz et al., 2009). The availableness and development of precise and subtle molecular techniques such as PCR-based fingerprinting for evaluating the diversity of root-nodule bacteria among closely related strains have highly influenced the research in this field. For example, REP-

PCR analysis has made it possible to distinguish between very related strains of rhizobia and other non-nodulating endopytic bacteria (Ming et al., 2008). Characterizations of chickpea nodule bacteria from diverse areas around the world have been conducted based on phenotypic and molecular methods (Maatallah et al., 2002; L'Taief et al., 2007; Tariq et al., 2012; Laranjo et al., 2014). Among molecular techniques that have been performed were DNA fingerprinting, restriction fragment length polymorphism (RFLP) and sequencing the conserved 16S rRNA genes (Maatallah et al., 2002; Rai et al., 2012). The results came from these approaches showed that the genera that can nodulate chickpea were diverse but they all belonged to *Mesorhizobium* genus. Also, chickpea nodules were found to harbor bacterial genera other than rhizobia and these non-rhizobial root-nodule endophytes can affect the plant growth and the nitrogen fixability of rhizobia as well.

In Turkey, chickpea (*Cicer arietinum* L.) is one of the important and first produced crops. However, in recent years, Turkey's chickpea production is in a downward trend (Aybegün et al., 2014). In Turkey, both economical and phytopathological problems have restricted the cultivation of chickpea and caused great decreases in its productivity. Turkish soils are nitrogen deficient and consequently nitrogen fixing root-nodule bacteria in addition to other nitrogen fixation helpers of NEB bacteria could promote yield, decrease production cost and maintain H_2O resources from NO_3 pollution. Besides, rhizobia and other NEB have been used to enhance the plant growth due to the excretion of growth-promoting hormones and also to increase the plant resistance against soil-borne fungi (Siddiqui and Akhtar, 2009; Küçük, 2013).

1.2 Objectives of The Study

Symbiotic nitrogen fixation by legumes is important as a practical means of saving nitrogen fertilization, improving the yield and quality of leguminous crops and indirectly maintains soil fertility. The increased use of rhizobia inoculants is to serve in accomplishing increased outputs of food and legume crops in the best saving way. So, much attention is required to discover new leguminous species with high production capabilities and high symbiotic performances. More effective strains of rhizobia will have to be discovered or developed and these "super" competitive strains will be more acceptable to their particular hosts than those currently in use. Then, perhaps it will be possible to bring about increased nitrogen fixation even in

soils which already harbor numerous highly infective strains of the microsymbiont. To get the best BNF out of any legume-rhizobium symbiosis, it is of importance to characterize and identify nodulating and non-nodulating bacteria before they are made commercially ready and applicable at fields (Sahgal and Johri, 2003). Wasike et al. (2009) reported that domestic strains exhibit diversity in their effectivity and competitiveness among hosts. The evaluation of the diversity among the natural communities of rhizobia and other NEB isolates in different geographical regions around the world has received an increased attention (Kücük and Kivanç, 2008; Dekak et al., 2018). Hence, the general objective of this study was to get a clear image about the diversity of chickpea root-nodule (nodulating and non-nodulating) bacteria in the agricultural soils in Turkey. The specific objectives of the proposal are:

a- Isolate and characterize root-nodulating rhizobia (RNR) of chickpea in the agricultural soils in Turkey on the basis of morphological, physiological and biochemical traits.

b- Isolate the chickpea non-nodulating endophytic bacteria (NEB) from the agricultural soils in Turkey in diverse geographical regions and characterize and identify them using diffent phenotypic traits and on molecular basis using REP-PCR and the 16S rRNA.

CHAPTER II

LITERATURE REVIEW

2.1 Rhizobia

The name "rhizobia" was first coined by Frank (1889) for root-nodule bacteria. Afterwards, all nodule-forming bacteria have been known as rhizobia. Rhizobia are microaerophilic, motile (flagellated), non-spore forming, soil-borne bacteria that multiply through cell division. Rhizobia via symbiosis with legume roots can fulfill the nitrogen demands of their host plants in a partial or complete degree (Sprent, 2007). They are major players in the BNF and the legume-rhizobium symbiosis provides for up to 450 Kg^{-1} ha $^{-1}$ of nitrogen per year (Unkovich and Pate, 2000). Somasegaran and Hoben (1994) described three stages in rhizobia's life including parasites, saprophytes and symbionts. During saprophytism, rhizobia live free in the soil without their host. Rhizobia populations that exist normally in a certain soil are known as native rhizobia, while rhizobia inserted into the soil via inoculation are said to be introduced (Abaidoo et al., 1999). Many diverse strains of rhizobia can exist within the native rhizobia populations. However, rhizobia populations that naturally exist in soil were found to be very low or not effective in many cases (Sanginga and Woomer, 2010). Also, rhizobia populations were reported to differ from one environment to another. For example, the *Bradyrhizobium* spp. strains isolated and characterized from African soybean cultivars were found to be different from the bradyrhizobia that nodulate with soybeans from North America (Abaidoo et al., 2000). Genetic diversity within indigenous strains was obviously put to the proof and was reported to vary between hosts (Wasike et al., 2009).

Conventional rhizobia were represented by the genera Ensifer *Mesorhizobium, Bradyrhizobium, Azorhizobium,* and *Rhizobium* (Sawada et al., 2003). However, in the last decade, other alphaproteobacterial genera like *Devosia, Ochrobactrum, Microvirga, Phyllobacterium* and *Methylobacterium,* were reported to be nitrogen-fixing root-nodule bacteria. Moreover, betaproteobacterial genera such as *Burkholderia* and *Cupriavidus* have been also reported as nitrogen fixers (Remigi et

al., 2016).

2.2 Legumes and Their Symbiosis with Rhizobia

Legumes are the 3rd biggest flowering plant family that has a huge number of different features, while a few characters are common to all species. Of these common characteristics is the ability to induce nodulation and form fixing nodules when associat to legumes in symbiosis. Symbiosis is a series of chemical interactions including the mutual specific signaling molecules which are coordinated and regulated to the highest degree between two partners (Fraysse et al., 2003; Oldroyd et al., 2011). The nodulation is initiated by the infection of root hairs due to the production of specific compounds such as dicarboxylic acids and flavonoids from the legume roots (Kape et al., 1991). In response to these compounds, rhizobia in turn, move by flagella to reach and attach to the legume root surfaces and the infection begins followed by root hair deformation. The attachment of rhizobia to roots is facilitated by polysaccharides that exist on bacterial cell surfaces and comply with the host plant lectins. Attached to the root surface, rhizobia induce root hair branching, deformation and curling to trap rhizobia in pocket-like structures (Fraysse et al., 2003). The cell wall of the roots is hydrolyzed due to degrading enzymes to help rhizobia get into the root hairs. Rhizobia inside the infection thread grow and migrate towards the nodule tissues in the inner cortex of the root where they are released into the host cell cytoplasm, then surround themselves by a protective membrane and differentiate into nitrogen fixing bacteroids. Nodules in which the atmospheric dinitrogen (N_2) is transferred into ammonia are known as effective and ammonia will be then assimilated by the plant and enhances growth especially in soils with nitrogen-deficiency. Rhizobia, in turn, are provided by carbon sources and using the nodule structure as shelters (Lodweg et al., 2003). On the other hand, N_2 is not fixed but rhizobia continue to get supplied with carbohydrates in case of ineffective nodules. This is seen as a sort of parasitism in the life cycle of rhizobia (Denison and Kiers, 2004).

Leguminous plants and rhizobia species show a degree of specificity to each other in their recognition, i.e. certain types of plants are colonized or recognized only by certain types of rhizobia. Some rhizobia have highly narrow host range and hence nodulate only certain specific plants that could be specific genus or species, while

others could have broad host spectrum. For example, *Rhizobium leguminosarum* bv. *viciae* has specific host range of genera; *Vicia, Pisurn, Lens* and *Lathyrus* (Stacey et al., 2006). Symbiotic nitrogen fixation by legumes is important as a practical means of saving nitrogen fertilization, improving the yield and quality of leguminous crops and indirectly maintains soil fertility.

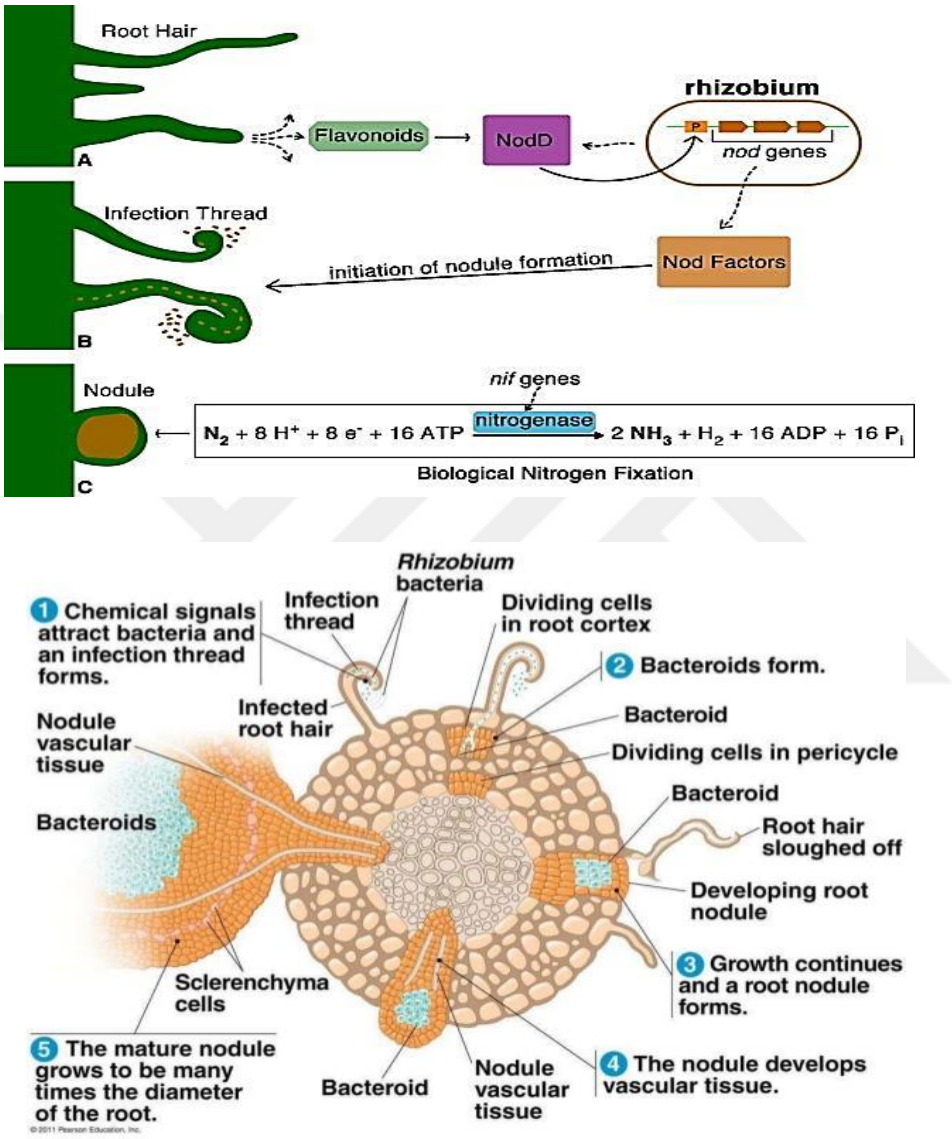


Figure 2.1 Chemical signals (A) (Laranjo et al., 2014) and steps of nodule formation (B) (www.slideshare.net/snehaljikamade/rhizobium-65788703) on legume roots

2.3 Importance of Biological Nitrogen Fixation in Agriculture

Nitrogen is not readily available for plant growth and must be reduced into a combined (fixed) form of NH_4 or NO_3 ions. Dinitrogen can be reduced through

industry via Haber Bosch process, or through BNF. During BNF atmospheric N_2 is reduced to ammonia in the presence of nitrogenase (Mulongoy, 1992). Fifty to seventy million tonnes of N_2 is fixed through symbiotic plants per year and contributes to the world agricultural N budget (Unkovich et al., 2008), which is equivaelnt to forty to seventy percent of the overall nitrogen input (Kahindi and Karanja, 2009).

BNF is a highly valuable resource for agriculture as its cost is much lesser than the synthetic fertilizers. So, it is considered the alternative to lower the reliance on manufactured fertilizers in agricultural systems. Via BNF legume plants reduce thirty to one hundred fifty kg of N_2 for each crop per hectare (Unkovich et al., 2008). BNF has both economic and environment merits over synthetic N-fertilizers. With respect to economy, it decreases production costs. Practical experiments have proved that the N_2 reduced by crops, due to rhizobia inoculants, costed $3/ha compared to $87 for fertilizer N_2 (Silva and Uchida, 2000). With reference to environment, using of rhizobia inoculants protects the soil from the contamination by the drain and release of extra fertilizers into water resources and thus decreases environmental problems (Kahindi and Karanja, 2009). Unlike BNF, ammonia fertilizers give rise to higher acidification of soils. Also, high levels of carbon dioxide are accumulated due to the synthetic reductions of N_2, which increases the global warming. Besides, BNF help decrease the nutrients leakage from soils. Studies have adduced that huge amounts of nutrients (660 kg N ha^{-1}, 75 kg P ha^{-1}, and 450 kg K ha^{-1}) have been lost during the last three decades from about two hundred million hectares of cultivated land in thirty-seven African countries (Sanchez et al., 1997). The legume-rhizobia symbiosis certainly offers the most promise in providing food and feed. It is assessed that leguminous plants fix 80^{10} tons yr^{-1} of N_2 from the atmosphere compared to 50^{10} to 60^{10} tons from synthetically manufactured N_2 world-wide (FAO, 2014). Increase in population numbers and the consequent increase in protein consumption is a challenge to provide for more high protein-rich food at low cost which opens the door for more reliance on BNF for survial.

2.4 Chickpea

2.4.1 Origin, Production and Associative Nodule Bacteria

Chickpea (*Cicer arietinum* L.) is one of the pioneer pulse crops that were

domesticated, and its origin goes back to south-eastern province of Turkey (Toker, 2009). It requires special environmental conditions of rain and humidity and thus prevails at arid and semi-arid regions of the world where moisture stress and low soil fertility exist (Gaur et al., 2008). Currently, chickpea is grown universally and it comes after dry bean as the most important food legumes (FAOSTAT, 2015). About 97% of chickpea cultivation is in the developing countries where it is used for the human and animal diet as an alternative protein source (Upadhyaya et al., 2007). It is rich in protein (having nutritious seeds whose protein content is 25-29%), complex carbohydrates, and fiber, while low in fat and cholesterol. The major producers of chickpea are India followed by Pakistan. They participate 68 and 9.75% of production, respecively. In addition to these two countries other countries like Iran, Turkey, Ethiopia and Syria are important contributors. Both living (fungal diseases, specially, fusarium wilt, and ascochyta blight) and non-living (temperature, salinity, and drought) limiting factors affect chickpea output and cause wide yield retard (Millan et al., 2006). Despite its great importance as a protein-rich source, only a few research works have been done on chickpea symbiosis. Chickpea is known to be nodulated by a narrow range of rhizobia (Broughton and Perret, 1999). BNF in chickpea contributes to 0-176 kg/ha of N_2 and this varies with rhizobia and biotic conditions (Beck, 1992). N-fixation and yield in chickpea can be promoted to higher levels via inoculation by *Mesorhizobium* inoculants (Beck, 1992). Chickpea is said to be a restrictive host in terms of nodulation and genera outside the genus *Mesorhizobium* can not induce nodules on its roots (Laranjo et al., 2014). *Mesorhizobium* has been described to induce nodulation, increase nutrient absorption, as well as increase chlorophyll contents of chickpea (Bejandi et al., 2012).

Currently, eight species of the chickpea microsymbiont *Mesorhizobium* have been described as capable of nodulating chickpea. Namely, *M. ciceri, M. mediterraneum, M. amorphae, M. huakuii, M. loti, M. muleiense, M. opportunistum,* and *M. tianshanense* (Jarvis et al., 1997). However, only three mesorhizobia species namely *M. ciceri*, *M. mediterraneum* and *M. muleiense* have a chickpea origin (Laranjo et al., 2014). It is worth mentioning here that a lot of agricultural lands that grow chickpea might be free of mesorhizobial species that represent the exclusive nodulating symbionts of chickpea. A clear example for such a condition is the western Canadian

soils which were devoid of indigenous rhizobia until introduced chickpea was inserted in 1990's (Kyei-Boahen et al., 2002). After being introduced, chickpea production has increased in this area; only introduced rhizobia were capable to induce nodules. This gives the rhizobia symbiosis and the inoculation processes the priority in the crop yield enhancement.

2.4.2 Turkish Chickpea and Its Nodule Bacteria

Cultivation and consumption of pulses in large quantities have been known since early times in agricultural areas around the world. In Turkey, chickpea contributes to about sixteen percent of the protein consumption (FAOSTAT-Agriculture, 2010). Moreover, Turkey is considred one of the pioneer countries with respect to domestication, production and exportation of chickpea. In 1984, Turkey produced one-third of the world's chickpea. However, in recent years, Turkey's chickpea production is in a downward trend (Aybegün et al., 2014). Fusarium wilt and ascochyta blight diseases are the main biotic stress factors negatively affecting chickpea yield in Turkey and throughout the world, causing up to 100% yield loss. Because most soils in Turkey are poor in nitrogen, therefore, rhizobia can enhance chickpea yield, decrease its production cost and save water resources from pollution by nitrates. Additionally, rhizobia can enhance growth by excreting growth-promoting factors (Laranjo et al., 2014) and increase the plant resistance against soil-borne fungi (Küçük, 2013). To improve chickpea inoculation effects, characteristics of indigenous rhizobia populations must be determined. The value of biological nitrogen fixation in Turkish agricultural lands and how it can flourish and increase the productivity of economically important crop plants like chickpea has not so far been appreciated and the need for inoculation of legumes is not clear. Also, very few studies paid the attention for the benefits of nitrogen fixation to the agricultural soils in Turkey (Hatice et al., 2008; Küçük and Kivanç, 2008). Although chickpea is cultivated in areas covering various provinces in Turkey, some regions are pioneers in its cultivation. The most producing provinces for chickpea in Turkey are central Anatolia and after that come southeastern Anatolia and mediterranean provinces. In general, the genus *Mesorhizobium* is the chickpea microsymbiont and two species of this genus were described as specific microsymbionts to chickpea; *Mesorhizobium ciceri* and *M. mediterranum*. However, at least 3 other species (*M. amorphae, M. loti* and *M. tianshanense*) have been identified to nodulate Turkish chickpea. Turkish

rhizobia that inoculate chickpea are paid little attention and few approaches identify them as *M. ciceri* (Hatice et al., 2008; Kütcük and Merih, 2008). For this purpose, isolation and characterization of various rhizobia is utmost important for symbiotic effectiveness.

2.5 Diversity and Taxonomy of Rhizobia

Native (natural) rhizobia reside in the soil during their saprophytic stage away from their macrosymbiont hosts. Populations of such naturally existing rhizobia can display diversity; having many different strains. The root-nodule bacteria strains are variable in accordance to environment. In the study by Abaidoo et al. (2000), it was reported that the *Bradyrhizobium* species that induce nodulation in an African soybean cultivar were found to be distinct and dissimilar to the North American soybean-nodulating Brabyrhizobia. Clear evidences were given for the genetic divergence among indigenous strains of root-nodule bacteria, and how these bacteria display their diversity in competitiveness and effectivity with and between hosts (Wasike et al., 2009). Several techniques were used for detecting and analyzing the diversity of rhizobia. Early researchers (Fred et al., 1932) used the word “rhizobia” as a single species to describe all bacteria that were able to induce nodule formation on all legume plants. Considering their growth rates on medium in the laboratory, Lohnis and Hansen (1921) distinguished rhizobia into 2 categories: the first was given the term "fast-growers" which commonly referred to rhizobia that displayed less than half the doubling time of the second category which in turn was identified as slow-growers. In the early 6^{th} decade of the nineteenth century, scientists began to use different characters in the numerical taxonomical studies of root-nodule bacteria, including nutritional, morphological, and biochemical traits, as well as, serology and simple DNA characteristics (Graham and Park, 1964). The ongoing research on the ability of root-nodule bacteria for nodulating different legume hosts has resulted in the development of cross-inoculation groups. In cross-inoculation rhizobia recovered from one plant were claimed to induce nodules on roots of all plants in the group (Fred et al., 1932). Sometimes, it happened that a single plant might be nodulated by more than one strain of bacteria, which indicates that the range of bacterial species and plant host is not necessarily tight. Cross nodulation was used to be a tool in rhizobial taxonomy until it was rejected and considered unreliable for taxonomic assays (Graham and Park, 1964). This rejection was partly due to anomalous cross-

infection between plant species. At the beginning attempts to study the diversity in rot-nodule bacteria, simple methods were utilized to distinguish strains within rhizobia species. Among those methods were; generation times, the ability of host plant to reduce nitrogen, tolerance to stresses of temperature, pH, salinity and resistance to antibiotics and heavy metals (Kamicker and Brill, 1986; Maâtallah et al., 2002; Ellis et al., 2003; Kücük and Kivanç, 2008). Moreover, the ability of bacteria to grow in the presence of different carbon sources and using these growth patterns have also been utilized to differentiate between isolates and strains of root-nodule bacteria. Additionally, total cell soluble protein patterns have been used to identify rhizobia strains and distinguish the sspecies within the same serogroup (Broughton et al., 1987; Fabriano and Arias, 1990; Kücük and Kivanç, 2008). Mostly, scientists utilize phenotypic-based characterizations to display a general view into the structure and diversity within rhizobia population. Such phenotypic characterizations serve as pilot differentiating tools to study the diversity of a given microbial population. Unlike phenotypic tools, molecular methods are used to determine the precise specific identity of the strains (Thies et al., 2001).

In the last few decades, several diverse molecular techniques have been routinely utilized for the precise identification of rhizobia diversity and they are common in laboratories world-wide. Examples for these techniques are plasmid profiling (Broughton et al., 1987), restriction fragment length polymorphism (RFLP) (Odee et al., 2002) and Polymerase chain reaction-based techniques (PCR) (Zhang et al., 2016). With the introduction of more DNA sequencing, there was a stepwise increase in the root-nodule genera and a clear image was obtained about the diversity of rhizobia and their relations with other bacteria groups inside and outside the nodules. Concurrently, as the methods of identification of root-nodule bacteria increase, there has been a parallel increment in the number of available published strains. Such an increase in the number of genera and species may be attributed to the raise in the numbers of legume plants that are being studied for nodulation. Despite of the increment in the number of the studied genera and species of legumes, only a small percentage (20%) of 18,000 species has been investigated for nodulation (Sprent, 2001). This means that still there are a huge number of legumes is waiting for investigation which means many more species and genera of rhizobia are expected to be identified.

Nowadays, new methods are improved to characterize and identify the root-nodule bacteria. For example, methods based on investiagting cell nucleic acids (DNA and RNA) have resulted in precise classifications of root-nodule bacteria. Hence, more genomes of the nodulating bacteria are made accessible which in turn enhances and promotes the taxonomical studies of these bacteria. According to recent researches, root-nodulating bacteria come with more than 100 species in more than thirteen genera (Weir, 2008). In addition to the tradtional genera of root-nodulating bacteria such as *Rhizobium, Azorhizobium, Bradyrhizobium, Ensifer* and *Mesorhizobium,* there has been described some new genera in alphaproteobacterial. The latter includes *Methylobacterium, Devosia, Phyllobacterium,* and *Ochrobactrum* (Sy et al., 2001; Rivas et al., 2003; Sawada et al., 2003; Trujillo et al., 2005; Radl et al., 2014; Zakhia et al., 2006). Over and above, betarhizobia genera have been found in the betaproteobacteria like *Cupriavidus* and *Burkholderia* (De Meyer et al., 2014; Remigi et al., 2016).

2.6 Non-nodulating Endophytic Bacteria (NEB) within Root-nodules

Although symbiosis between nodulating bacteria and the host legumes are of high specificity and selectivity, there was reported the presence of non-nodulating rhizobacteria inside nodules. Root-nodules were thought to be exclusive to only genera of rhizobia. However, recent works in the last decades made it clear that also numerous non-nodulating genera can coexist alongside with rhizobia inside the nodules. Such bacteria are generally given the term non-rhizobial endophytic bacteria (NEB) and they perform many functions not necessarily connected to symbiosis or nitrogen fixation (De Meyer et al., 2015; Zhao et al., 2018). Beijerinck and Delden (1902) reported for the first time the existance of NEB inside root nodules and the first identified genus of such nodule endophytes was *Agrobacterium radiobacter*. These non-nodulating NEB bacteria can go into the interior of nodules through the joint between nodule and the root. Recently, Zgadzaj et al. (2015) stated that NEB bacteria can enter the nodules via the infection thread created by rhizobia. The term “root-nodule endophyte” was used in our study to describe bacteria that exist within the chickpea nodules but are not able to trigger nodulation.

Non-nodulating nodule endophytic bacteria exist in 3 classes α-, β- and γ-Proteobacteria as well as some Actinobacteria and Firmibacteria. α-Proteobacteria

class include numerous NEB genera such as *Devosia, Aminobacter, Shinella, Ancylobacter, Caulobacter, Phyllobacterium, Sphingomonas* and mnay others. β-Proteobacteria also have many non-symbiotic endophytic genera like *Massilia* and *Variovorax, Bordetella* and others. Similarly, the third class γ-Proteobacteria includes important genera of NEB like *Acinetobacter, Enterobacter, Pantoea, Pseudomonas, Serratia* and others. Besides, there are some NEB members belonging to actinobacteria like *Arthrobacter, Curtobacterium, Microbacterium, Mycobacterium, Nocardia* and *Streptomyces* (De Meyer et al., 2014). Above that, members of NEB are also included in the genera Staphylococcus are *Bacillus,* and *Cohnella* (Tariq et al., 2012).

Also, researches have described a great diversity of leguminous plants that host the NEB bacteria. These host legumes include alfalfa, chickpea, clover, common bean and others (Dudeja et al., 2012). Some NEB endophytes render useful services to the host plants and excreting plant growth promoting factors is the clearest exemplar of beneficial impact of NEB on host plants (Tariq et al., 2014). Also, tasks other than growth promotion can be served by the nodule-NEB bacteria; these include nitrogen reduction, increment of host stress tolerance against soil-borne microorganisms and biological control of plant pathogens (El-Tarabily et al., 2010). Various research works investigated the useful impact of inserting these NEB endophytes (as inoculants) with rhizobia to improve the host growth and tolerance (Andrews et al., 2010; Egamberdieva et al., 2010).

2.7 Phenotypic Characteristics of Root-nodule Bacteria (RNB)

Phenotypic characterizations of culture characteristics of microorganisms have the advantage of being fast and allowing a preliminary analysis of diversity. They are important for getting a general idea about the expected variation inside a bacterial population, predicting a complementary genetic variation within the population and selecting candidate isolates that might tolerate harsh abiotic conditions.

2.7.1 Cultural and Morphological Characteristics

Rhizobia are gram-negative bacteria that appear as short rods under microscope (Somasegaran and Hoben, 1994). The growth rate of legume-associated rhizobia on yeast-extract mannitol agar medium was used to categorize rhizobia as very fast, fast,

intermediate and slow (Odee et al., 1997). Meanwhile, in Bergey's Manual, the major genus *Rhizobium* in the family of Rhizobiaceae was reported to be fast-growing on YMA (Jordan, 1984). Rhizobia have generation time of 2 to 4 hours in YMA medium and their colonies were with 2 to 4 mm in diameter within 72-120 hours of incubation (Graham et al., 1991; Somasegaran and Hoben, 1994). It was reported about most rhizobia that they do not or slightly absorb the congo red dye supplemented to the growth medium. Also, it is known that fast-growing rhizobia produce acids that could turn the dye into purple (Somasegaran and Hoben, 1994). Early and recent studies have used cultural and morphological characteristics as phenotypes to characterize rhizobia including growth rates and colony characteristics on YMA medium (Graham et al., 1991). More than four hundred eighty rhizobia isolates were collected and investigated by Odee et al. (1997) from nodules of woody and herbaceous leguminous plants from twelve sites in the Kenyan soils. Their isolates, in major part (91%), were developed colonies with clear watery phenotype. The remaining isolates were creamy or white opaque. Recently, many works have also utilized the morphological phenotypes to characterize rhizobia and NEB bacteria inside the nodules of many hosts (Kücük and Kivanç 2008; Aserse et al., 2013; Rai et al., 2013; Khalifa et al., 2016).

2.7.2 Nutritional Requirements

2.7.2.1 Carbon and Nitrogen Utilization

The capability of root-nodule rhizobia to metabolize different sugars as sole carbon sources was used as a discriminative tool to distinguish species of rhizobia. Also, significant differences among rhizobia strains were reported regarding their utilizations of carbohydrates as carbon sources (Somasegaran and Hoben, 1994). Fast-growing rhizobia, due to their possession of a dehydrogenase activity, were described to use a wider range of carbon sources than do other genera of root-nodule bacteria (Zhang et al., 1991). Unlike rhizobia, bradyrhizobia lack the activity of such a dehydrogenase activity, but still yet metabolize many carbon compounds. About chickpea symbionts, observations from many approaches described them to use numerous carbohydrates (L'taief et al., 2007). Also, in the study by Maâtallah et al. (2002), they found that all chickpea isolates were able to grow with numerous carbohydrates including mannitol, surose, sorbitol, trehalose, and fructose while none

of the isolates utilized xylose, glycogen or inulin. In their study of the biochemical characteristics of bean rhizobia, Kücük et al. (2006) reported the ability of isolates to metabolize different compounds as sole carbon source. The whole isolates grew with galactose, mannitol, sucrose and strach while no growth was observed for dulcitol and tartrate. Also, rhizobia from chickpea were investigated by Kücük and Kivanç (2008) for their growth with twelve carbon sources and the isolates recorded positive results for all the carbon sources. Similar results were reported for non-nodulating (NEB) bacteria as they were also found to utilize numerous carbohydrates (Demissie et al., 2018).

Similarly, the interaction between nitrogen fixation and the available mireral N in soil is of a major practical importance. It is widely accepted that the addition of combined nitrogen to the soil reduces the potential of the legume to fix N. Among nutritional requirements of rhizobia and non-nodulating nodule endophytes is the utilization of nitrogen sources. Rhizobia can metabolize inorganic nitrogenous compounds (e.g. nitrate and ammonium salts), short chain peptides and amino acids to meet their needs of nitrogen (Jordan, 1984). Chickpea root-nodule bacteria could utilize various nitrogen sources (Jida and Assefa, 2012). Certain amino acids as glycine (Jordan, 1984), asparagine and L-methionine (Zhang et al., 1991) may be inhibitory for rhizobia growth. However, Mohamed et al. (2000) found that the latter two amino acids can be utilized by some rhizobia isolates from *Acacia* spp. as a sole nitrogen source.

2.7.3 Salt Stress

Near about forty percent of the land surface and twenty percent of the cultivated land world-wide, especially tropics and mediterranean regions, were reported to have salinity problems which lead to the waste of half the agricultural yield (Gamma et al., 2007). Salinity inhibits the proliferation and activity of root-nodule bacteria through toxicity and osmotic stress (Singleton et al., 1990). Reduction in both respiration and production of cytoplasmic proteins in root-nodules (leg-haemoglobin in particular) was reported to be a result of salinity. This leads to inhibition of the early stages of infection process, affecting root-nodule developments and efficiencies, and reducing the host growth (Tate, 1995). Rhizobia adaptation to salt-stress was attributed to the assemblage of certain low organic solvents (osmolytes such as glutamate,

glycinebetaine and proline) of low molecular weight inside cells to oppose the water-removing effect of low water activity in the nodule medium. Such osmolytes normally do not interfere with other structural molecules necessary to the organism (Smith et al., 1994). Rhizobia are more tolerant to salt than the legume plants and there is variability in their salt tolerance. To get effective symbiosis between rhizobia and legumes in saline conditions, it is important to select tolerant rhizobia as well as the host legume (Saadallha et al., 2001). However, under salt conditions, the legume host tolerance to salinity is the main and most effective factor that determines the success of rhizobia strain to form a successful symbiotic relation with the the host legume (Craig et al., 1991). It is generally observable that fast-growing rhizobia strains are more salt tolerant than slow-growers (El-Sheikh and Wood, 1995). In saline soils, salt-tolerant strains of rhizobia can serve in the rehabilitation process. Therefore, survival of rhizobia under saline laboratory media is valuable in choosing candidate strains that can trigger effective symbiosis in saline soils (Singleton et al., 1990). Similarly, salinity tolerance among root-nodule bacteria has been used as a characterizing tool in early and recent works. It varies from species to another. For example, strains of *R. meliloti* were found to tolerate 100 mM, while *R. leguminosarum* tolerated up to 350 mM NaCl in broth culture (Mashhady et al., 1998; Abdel-Wahab et al., 2002). Rabie and Alamadini (2005) observed that low and moderate salinity levels did not affect the growth of rhizobia. Also, Küçük et al. (2006) demonstrated rhizobia strains that grew variably at high salt concentration (5%). Observations also were recorded for NEB as tolerate different salt levels (Khalifa et al., 2016).

2.7.4 Heat Stress

Temperature conditions have a great effect on growth of root-nodule bacteria. Niste et al. (2013) reported that growth of root-nodule bacteria was highly affected by thermal conditions. High temperatures strongly affect the rhizobia infection, nitrogen fixation and plant production (Hungria and Franco, 1993). Nodule initiation and nodulation are also affected severely by high temperatures (Graham, 1992). Infection thread and bacteroid developments are found to be also affected by high temperature (Hungria and Vargas, 2000). In addition to physiological modifications, genetic alterations in rhizobia including plasmid deletions and genomic rearrangements can be affected by high temperatures. This may result in alterations or losses of

symbiotic properties. Also, a sudden temperature change causes formation of heat shock proteins (HSP), and cold shock protein (CSP) that help root-nodule bacteria to survive (Yura et al., 2000).

Most strains of rhizobia were described to grow optimally in the cultures at a range of temperatures between 28 to 31°C, while 37°C did not support the growth of many species (Graham, 1992). However, most strains of *R. leguminosarum* studied by Karanja and Wood (1988) found to persist at 45°C, but they were ineffective in terms of nitrogen fixation due to plasmid curing. Despite that, some chickpea rhizobia have shown their maximum growth at 20°C (Rodrigues et al., 2006). Also, Caudry-Reznick et al. (1986) observed the growth of arctic rhizobia at 10°C. The tolerance of rhizobia and NEB bacteria to heat stress was reported to be variable among genera. For example, in the study by Maâtallah et al. (2002), most isolates from chickpea nodule bacteria showed maximum growth at 20-30°C. Below and above this limit, the growth was significantly decreased (12% at 5°C and 7% at 45°C). And above 45°C, more than half the isolates were not able to withstand. Also, in a study on root-nodule bacteria from lentil, Moawad and Beck (1991) found that *R. leguminosarum* isolates were tolerant to temperatures between 35-40°C, but the symbiosis was less effective. Similarly, thermotolerant rhizobia strains were observed in 90% of isolates from cowpea as they endured at 40°C (Eaglesham and Ayanaba, 1984). Also, despite their inability to form effective nodules, most *R. phaseoli* srains, from *Phaseolus vulgaris*, survived at 45°C (Karanja and Wood, 1988). Most of the isolates from *Acacia* studied by Mohamed et al. (2000) survived at 35-37°C and only few strains endured above 40°C. The most thermotolerant strains were isolated by Kulkarni et al. (2000) from *Sesbania aculeata*. Only 2 strains in their study survived at 50-65°C in broth culture for up to 2-4 hrs. In the study by Hung et al. (2005), many of their strains grew well at 37-45°C and in the study on rhizobia from bean by Kücük et al. (2006), plentiful growth at 42°C was observed. Also, Kücük and Kivanç (2008) studied the temperature tolerance in rhizobia from chickpea and all their strains were able to survive at 20- 37°C, and 75% were tolerant to up to 40°C. In addition, Most of the isolates studied by Rodrigues et al. (2006) showed best growth at 28°C while 20°C was the optimal temperature for only 1 isolate.

2.7.5 pH (acid/alkali) Tolerance

Among the main limiting factors for the growth of several microorganisms in soil is the pH value (Brockwell et al., 1995). There are some factors that can affect the pH in a particular habiat. Generally, increment in some minerals like Al and Mn was found to be associated with low soil pH. Also, deficiency in Ca^{++}, PO_4^- and Mo can decrease the pH in soil (Hungria and Vargas, 2000). Graham et al. (1994) reported that tolerance to pH within root-nodule bacteria can be associated with the internal chemical composition of rhizobia. For example, Brockwell et al. (1995) observed that accumulation of cellular polyamines, lipopolysaccharide composition and acid shock proteins can increase the pH tolerance and give advantages to the strains in which such chemical changes are expressed.

It has been reported that fast-growing rhizobia with fast growth rates were described to have less tolerance to low pH values than slow-growers which were considered to be more tolerant to acidic pH values. Also, the differences in tolerance to pH among fast- and slow-growing rhizobia are not attributed to clear reasons (Correa and Barneix, 1997). However, tolerance to acidic pH values was reported for many species of the genus *Rhizobium*. For instance, *R. trifolii, R. loti,* and *R. tropici*, (Graham et al., 1994). The optimum pH in rhizobia was described to differ from a strain to another within a given species. Generally talking, pH 6-7 was recorded as the optimum range for rhizobia growth (Tang and Thomason, 1996). For instance, different strains of *Bradyrhizobium* spp. were assessed by Raza et al. (2001) for tolerance to pH values and they tolerated a wide range of pH (4-10). Similarlly, effective chickpea rhizobia isolated from different regions in Morocco were found to tolerate pH 5-8 (Maâtallah et al. 2002). Besides, *Rhizobium* strains from *phaseolus* were tested for tolerance at pH (4-9) in YMA medium (Kücük et al., 2006). All the isolates grew well at pH 5-8 and they all showed alkaline tolerance (all grew at pH 9), but variations were observed at pH 4. In addition, the study by Rodrigues et al. (2006) on growth of rhizobia at pH (5-8) showed a positive relation between the soil origin, where isolates were collected, and the maximum growth pH. Kücük and Kivanç (2008) investigated the growth of rhizobia from chickpea in YMA with different pH and observed that pH 5-8 supported the growth of all isolates and differences were detected at pH 3 and 9. Additionally, most of the isolates from *Acacia* spp. in the study by Mohamed et al. (2000) were able to tolerate pH at both

acidic and alkaline values. Shamseldin and Werner (2005) found that most of their isolates grew minimally at pH 4.7. Also, growth at highly acidic and alkaline pH values of root-nodule bacteria was also detected. For instance, most isolates studied by Hung et al. (2005) tolerated extreme pH between 3.5-12. Moreover, except for 3 isolates which were able to survive at pH 12, most isolates in the study by Kulkarni and Nautiyal (1999) showed good growth at pH 9.

2.7.6 Antibiotic Resistance

Diversity within rhizobia strains of the same species has been investigated through studying the pattern of their antibiotic resistance. A clear example for this was the study by Karanja and Wood. (1988) who tested the strains of *R. leguminosarum* bv. *phaseoli* for resistance to some antibiotics including streptomycin, rifampicin, ampicillin, spectinomycin, nalidixic acid, genomycin chloramphenicol, and tetracycline at diferent concentrations. They found that, there were differences among strains for resistance to antibiotics. Tetracycline inhibited the growth of all rhizobia, and 20 µg ml^{-1} of rifampicin, spectinomycin or genomycin were enough to inhibit growth for more than 93% of the *Rhizobium* strains tested. Ampicillin and nalidixic acid had the lowest inhibitory effect on rhizobia. Sensitivity to different antibiotics, at different ranges, of concentrations, varied between species and such variation may be a useful taxonomic character (Somasegaran and Hoben, 1994; Jida and Assefa, 2012). Fast-growing rhizobia strains were found to be more susceptible to antibiotics than slow-growers (Jordan, 1984). In line with this, Mpepereki et al. (1997) studied the antibiotic sensitivity of cowpea (*Vigna unguiculata*) nodule isolates from 14 Zimbabwean soils and found that the intrinsic antibiotic sensitivity was generally higher in fast-growers compared to slow-growers. For example, Gupta et al. (1983) used the intrinsic antibiotic resistances to invistigate root-nodule bacteria of mung bean. Their tested antibiotics (µg $disc^{-1}$) were erythromycin (15) ampicillin (10), tetracycline (30), gentamycin (10), streptomycin (10), kanamycin (30) and chloramphenicol (30). The pattern of antibiotic resistance clarified that a large number (53%) of natural rhizobia were susceptible to all the evaluated antibiotics. On the other hand, there were many findings that fast-growing root-nodule bacteria (Like chickpea nodule bacteria) could show resistance against a variety of antibiotics. For example, Maâtallah et al. (2002) studied intrinsic resistance of chickpea bacteria to different concentrations (µg ml^{-1}) of the following

antibiotics; ampicillin (50), chloramphenicol (10), kanammaycin (10 and 100), rifampicin (10), streptomycin (25 and 100), nalidixic acid (50), erythromycin (100) and tetracyclin (20). Results revealed that 65% of the isolates showed high insensitivity to kanamaycin, nalidixic acid, and erythromycin. While 14-25% were insensitive for streptomycin, ampicillin, chloramphenicol, and tetracyclin. The results led to the conclusion that tolerance to antibiotics was related to the species of bacteria, but not to their growth rates. Kücük and Kivanç (2008) utilized different concentrations of many antibiotics including streptomycin, kanamycin, erythromycin, chloramphenicol, and penicillin. Most strains were found to be highly insensitive against the assessed antibiotics.

2.7.7 Effect of Heavy Metals on the Survival of Rhizobia

It was reported from investigations that not only the growth, but also the morphology and the activities of microorganisms are adversely affected by heavy metals. It has been reported that the number and/or activities of soil microorganisms were reduced in soils contaminated with heavy metals. Several studies had shown that heavy metals might have been toxic to rhizobia when present in soil in moderate to high concentrations (Giller et al., 1989). In addition, heavy metals influenced protein profiles of rhizobia, by decreasing polypeptide expression in sensitive strains and increasing in resistant strain (Pereira et al., 2006). Like antibiotics, the tolerance to heavy metals could serve as a valuable marker in the genetic studies of the rhizobia isolates (Küçük and Kıvanç, 2008). Also, tolerance to heavy metal toxicity on synthetic media might be related to their tolerance at their habitats. For example, Biomy (2000) concluded that the isolates of rhizobia from *Vicia. faba* grown in sewage sludge-contaminated soils were highly resistant to heavy metals than other strains. Furthermore, the high level of resistance to certain heavy metals suggests that such metals might be utilized as a selective tool for resistant bacteria strains (Sinclair and Eaglesham, 1984).

2.7.8 Biochemical Activities of Root-nodule Bacteria

Although being indecisive in the precise identification of root-nodule bacteria, biochemical activities have been used as one of the characterizations tools to get a general image about the diversity of root-nodule bacteteria. Numerous biochemical tests have been employed to assess the diversity of nodule bacteria isolated from

different plants (Naz et al., 2009; Sharma et al., 2012; Niste et al., 2015; Khalifa et al., 2016). For instance, in the study by Küçük et al. (2006) the biochemical activities of thirty isolates of bean nodules grown in Eskişehir, Turkey, were investigated. Also, Kaur (2014) utilized the biochemical characteristics of chickpea nodule isolates to investigate their diversity in agricultural soils in India. The results revealed that the isolates displayed positivity for catalase, oxidase, citrate, and nitrate reduction, while negative records were obtained for ketolactose and methyl red tests. Similarly, in chickpea root-nodule bacteria, positive responses to oxidase, citrate and catalase were reported (Gauri et al., 2012; Singh et al., 2013; Wani and Khan, 2013). Besides, different biochemical tests were done by Roychowdhury et al. (2015) which included methyl red (MR), H_2S, catalase, nitrate reduction and amylase tests. Results showed positive MR and catalase while they were negative for nitrate, amylase and H_2S. Moreover, Singha et al. (2015) investigated 14 isolates from root nodule of *Crotolaria junceae* from 4 different sites of India, for seven different biochemical characteristics namely oxidase, nitrate reduction, catalase, starch hydrolysis, urease, citrate and gelatin liquefaction test. Most of the isolates gave positive observations for oxidase, nitrate reduction, catalase, and urease whereas negative results were obtained with starch hydrolysis, citrate utilization and gelatin hydrolysis test. Among the biochemical activities of root-nodule bacteria was their ability to excrete the phytohormone indole acetic acid (IAA). Rhizobia isolates from many host plants were described to produce IAA (Perrine et al., 2004; Kumari et al., 2009). It was reported that the production of IAA by root-nodule bacteria strains can serve to stimulate the host plant growth (Perrine et al., 2004; Verma et al., 2013).

2.8 Molecular Characterizations of Root-nodule Bacteria

The characterization of root-nodule bacteria has been traditionally based on phenotypic characters, but this approach has been widely considered as not adequate (Eardly et al., 2005). It has been argued (Demezas et al., 1991) that reclassification of the genus Rhizobium should be carried out based on molecular characterization, rather than of rhizobial plant host specificity or other phenotypic characteristics. In general, phenotypic methods serve to predict a complete point about structure and diversity of strains inside a population of strains. They are primarily discriminatory method for symbiotic studies. However, molecular methods are more preferred to describe the specificity of the strains (Thies et al., 2001). At present, many molecular

methods are frequently used in all labs world-wide to determine the identity of bacteria from root-nodules. Highly accuarate characterizations are currently made available due to the ongoing progress in the DNA and RNA methods. Hence, a parallel increment in the available number of total bacterial genomes has been achieved which has a major impact on root-nodule bacteria characterization and identification.

2.8.1 Characterization of Root-nodule Bacteria Using REP-PCR

Researches identified the existence of repetitive sequences along the DNA in the genome of soil-borne bacteria such as nondulating and non-nodulating nodule bacteria (Stern et al., 1984). These dispersed sequences are known as REP- elements or units (repetitive extragenic palindromic). Since they have highly conserved palindromic sequences, they were expected to represent a useful fingerprinting tool to identify the bacterial genome (Versalovic et al., 1991). When the PCR amplıfıcatıons were used to detect and amplify these REP-units via specific primers, easily resolvable bands in electrophoresis were obtained in a reproducible way. The patterns of the obtained bands for REP-elements provided a tool for fingerprinting and analyzing the genomes of different species and strains within the investigated bacterial genera. This, in turn, facilitates and fastens the identification process of a bacterial collection (Mullis and Faloona, 1987). Also, sequences of REP-elements have been described in various bacterial strains. In PCR amplifications of REP-units, the specific primers anneal at specific sites corresponding to these repeat sequences on the template gDNA. This allows the primers to amplify these sequences to yield patterns that were found to be unique to each strain (Versalovic et al., 1991).

Many recent works have used this technique as the molecular choice for characterizing rrot-nodule bacteria (RNB) starins. For example, Zhang et al. (2016) characterized the intra-species diversity of rhizobia in soybean rhizospheres by repetitive extragenic palindromic PCR. Also, Ezzakkioui et al. (2015), via PCR amplifications of REP-elements, clustered 70 bacterial strains from *Hedysarum* nodules collected from Moroccan soils. REP-PCR band patterns put these isolates into thirty groups. Also, Mishra et al. (2017) through REP-PCR amplifications, clustered 15 isolates of *Bacillus* collected from root-nodules of 6 legumes including soybean, pea and lentil into clearly resolvable band paaterns. Besides, Tajima et al.

(2000) used REP-PCR to fingerprint Japanese isolates of different genera including *Bradyrhizobium*, *Sinorhizobium*, and *Rhizobium*.

2.8.2 Identification of Root-nodule Bacteria Using 16S rDNA

When Carl Woese introduced the analysis of 16S rRNA sequence for studying the phylogeny of microbial populations, it was a great, and might be the most important, step towards the precise identifications of microbes (Woese, 1987). The 16S rRNA gene sequence analysis supplies obvious undoubtful data for the identification of even very scarce strains in opposition to the phenotype-based characterizations which might be doubtful due to the variation in the expression of traits. Also, the data from 16S rRNA analysis are reproducible among labs world-wide. 16S rRNA genes exist in all livings including eukaryotic and prokaryotic organisms. These organisms contain these genes with many highly conserved sequence patterns rendering the same functions. Such sequence patterns can be used to differentiate microorganisms that belong to different phylogenies through comparing the differences in the 16S rRNA gene sequences and match the sequences with the pre-identified data. Therefore, 16S rRNA gene nucleotide sequencing is a good robust reproducible tool to study and identify the evolution changes among species of a given population. At present, classification sequencing of nucleotides of the 16S rRNA gene is the base for a plethora of taxonomic investigations of different microbial communities. Due to its universal applicability and reproducibility, 16S rRNA resulted in a huge database of highly conserved sequences that were made available worldwide via research publications. This enables the scientists to investigate the novelty of new identified isolates by analyzing and comparing the sequences of new identified species with sequences of already known strains and build up predictable taxonomical evolutionary relationships among the isolates. This is easily done through computer-based informatics which can detect the similarities in sequences between the investigated sequences and each sequence in the world database and put the similarity in a percentage of relatedness to the nearest possible identity. Such comparisons of sequences are achievable online via numerous gene databases (e.g. National Center of Biotechnology and Information-NCBI) by applying Basic Local Alignment Search Tool (BLAST). A BLAST search enables researchers to compare a query sequence with a library or database of sequences and identify library sequences that resemble the query sequence above a certain threshold. This is done

through calculating an optimal alignment between the sequences without allowing gaps in the alignments. The world-wide usage of 16S rRNA has its useful shadows on the research field of root-nodule bacteria and thousands of research papers on rhizobia and other nodule endophytic bacteria have been described based on 16S rDNA gene sequencing technology (Aserse et al., 2013; Zhao, 2018). For instance, Aserse et al. (2013) utilized the 16S rRNA sequencing analysis to precisely determine the taxonomical positions and identify fifty root-nodule isolates from different legumes in thirty-one different sites in the agricultural soils in Ethiopia. Also, Saidi et al. (2011) investigated the diversity and identity of 104 root-nodule bacteria of faba bean from agricultural soils in Tunisia using the analysis of 16S rRNA gene sequencing.

CHAPTER III

MATERIALS AND METHODS

This research study was accomplished at the Department of Microbiology, Faculty of Science and Arts, Gaziantep University, Gaziantep, Turkey. The materials and methods were as follows:

3.1 The Study Sites

A total of 133 bacterial isolates from chickpea (*Cicer arietinum* L.) root-nodule were collected during surveys covered 17 cities representing six provinces in Turkey. These regions covered all the main provinces which are important in chickpea cultivation in Turkey. The attributes of GPS were used to detect the sites where the chickpea host plants were collected for the sake of isolation and characterizations (Woomer et al., 2011). The descriptions of the collection sites were given in table 3.1 and shown on map in Fig. 3.1.

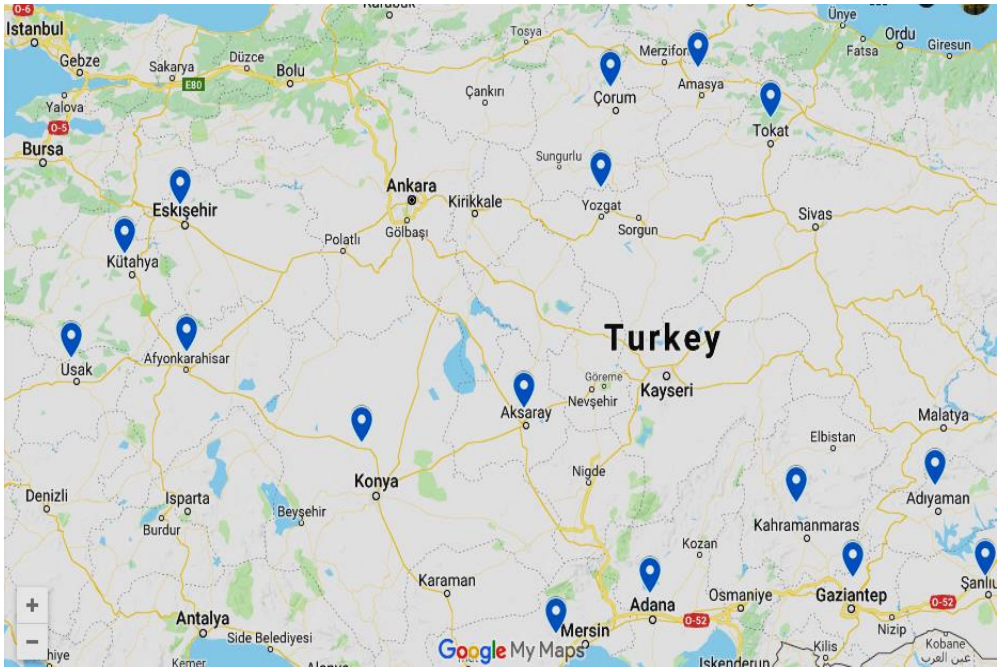


Figure 3.1 Collection sites of chickpea root-nodule bacteria in Turkey

Table 3.1 Chickpea root-nodule bacteria used in this study and their collection sites

Origin	GPS Attributes		Isolates
Adana	N[36°05'24"]	E[37°18'41"]	Ada2/Ada5A/Ada6A/ Ada8/Ada9/Ada10
Adıyaman	N[37°35'02"]	E[37°58'57]	Adi1/Adi4/Adi5/Adi6/Adi7/ Adi9/Adi12A/Adi14/Adi15/ Adi16A1/ Adi17
Afyon	N[38°25'43"]	E[30°38'18]	Afy1/Afy3/Afy5
Aksaray	N[38°31'43"]	E[34°24'42]	Aks1/Aks2/Aks3A/Aks3B/ Aks4A/Aks5/Aks7/Aks10
Amasya	N[40°34'47"]	E[36°19'06]	Amas3/Amas4/Amas7
Çorum	N[40°21'34"]	E[34°51'14]	Cor2A/Cor3/Cor4/Cor5/Cor7/ Cor9
Eskişehir	N[39°30'03"]	E[30°45'13]	Esk6/Esk7A/Esk7B
Gaziantep	N[37°16'11"]	E[37°24'03"]	Gbak/Gbur1A/Gbur1B/Gbur3/ Gogu1/Gogu2/Gsar1A/ Gsehinb/Gsehit1Gsenlik1/Gsut2/ Gyav1/ Gyav3A/Gyes1
Hatay	N[36°2 '43"]	E[36°16'36"]	Hat1/ Hat2/ Hat3/ Hat4A
Kahraman	N[38°22'54"]	E[36°54'34"]	Kah1/Kah2A/Kah3/Kah6/Kah7/ Kah8/Kah9/Kah10/Kah13
Kırşehir	N[39°19'27"]	E[33°57'16"]	Kir1/Kir4/Kir5A/Kir9/Kir10/ Kir12/Kir15/Kir17A/Kir19/ Kir20/Kir21
Konya	N[37°43'51"]	E[31°46'32"]	Kon2/Kon3/Kon7/Kon9
Kütah	N[39°16'11"]	E[30°10'40"]	Kut2/Kut9
Mersin	N[36°29'42"]	E[33°58'06"]	Mer1/Mer2/Mer3/Mer4/Mer5A/ Mer5B/Mer6A/Mer6B/ Mer7/Mer8/Mer9/Mer10A/ Mer11/Mer12/Mer13/Mer16/ Mer17/Mer18/Mer26
Tokat	N[40°15'32"]	E[35 46'58"]	Tok1/Tok2/Tok3/Tok4
Urfa	N[37°03'13"]	E[38°11'42"]	Urfa2
Usak	N[38°31'20"]	E[29°26'44']	Usak1/Usak2/Usak13/Usak17
Yozgat	N[39°51'13"]	E[34°57'19"]	Yoz2/Yoz6/Yoz7/Yoz8/Yoz11/ Yoz14/Yoz15/Yoz16

3.2 Isolation of Root-nodule Bacteria

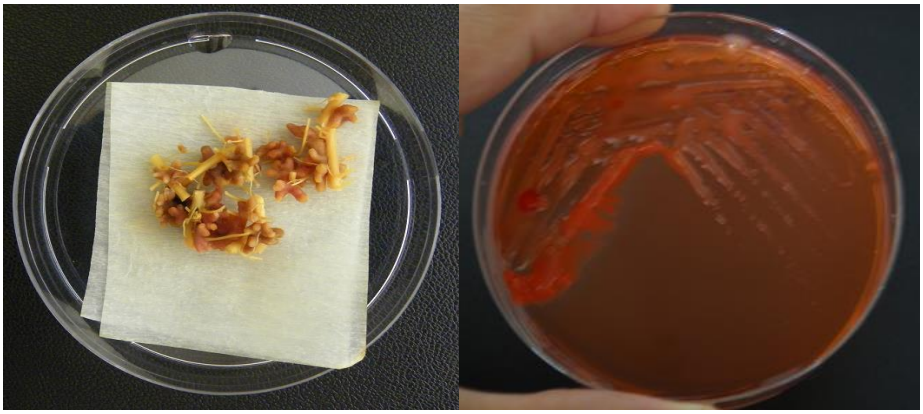
Healthy chickpea plants were taken from soil carefully without breaking or cutting the lateral roots due to their having nodules useful for isolation. Along with small parts of the lateral roots, nodules were excised to prevent air from getting into inside the nodules through the junction between nodules and the roots. Nodules were then transferred to the lab on absorbents (e.g. silica gel) to keep them dry (Woomer et al.,

2011). Undamaged nodules were surface sterilized with 95% ethanol for 5-10 minutes, and then they were soaked in a 3% solution of sodium bleach for 4-6 min to asure surface sterilization. Then, nodules were washed several times in sterilized dH_2O (Vincent, 1970). Dry nodules must be re-hydrated before sterilization. Desiccated nodules were left to absorb sterilized H_2O for 2 hours at room temperature. Afterwards, surface sterilization nodules were grinded or cut with a sterilized cutter in a drop of sdH_2O. About 10-20 µl of the nodule sap was distributed on the selective solid media of yeast-extract mannitol agar (YMA). This YMA medium comprises these amounts in grams; 10, 0.5, 0.2, 0.1, 0.5 for mannitol (C-source), K_2HPO_4, $MgSO_4.7H_2O$, NaCl, and yeast extract (N-source), respectively. For solid media, agar is added 15g $L^{-1}$and the pH is set, before autoclaving at 121°C for twenty minutes, to be 6.8 via NaOH and HCl (Vincent, 1970). To easily distinguish the rhizobia colonies from other possible contaminants, the congo red dye is added to YMA medium to make a final concentration of twenty-five part per million. This dye is not (or slightly) absorbed by rhizobia whereas other contaminants take in the dye and thus appear heavily pink or red on YMA medium. After autoclaved, the YMA media were left to cool down to 55°C and poured to Petri-dishes. For incubation, YMA cultures with nodule extracts were left for 3-5 days at 30°C. For the isolation of non-nodulating endophytes and to guarantee that the interior of nodules was the source for the colonies of NEB bacteria, few drops of the sterile distilled water (used in the final sterilization rinse) was streaked onto YMA cultures and incubated for 5 days at 30°C. If any colony growth was observed this meant that the process of disinfection was not perfect, and the sterilization was repeated. Plates without any contaminants were considered effectively surface sterilized and their YMA plates were used for the isolation of endophytes.

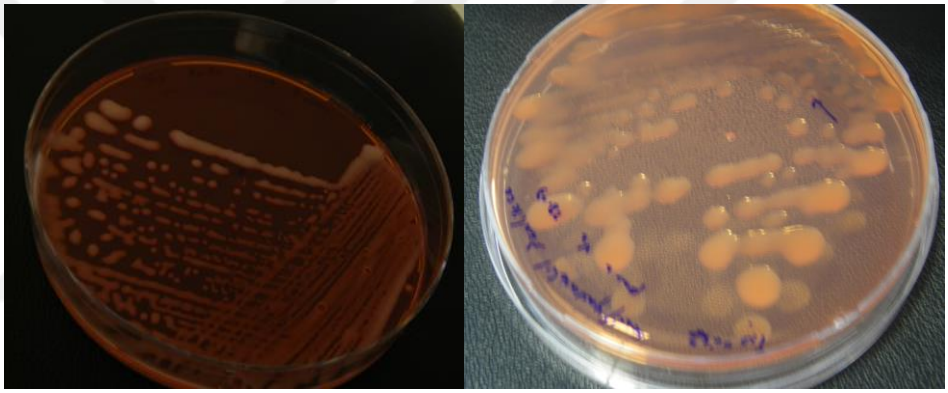
3.3 Purification of Root-nodule Bacteria

For a perfect purification of cultures, a single colony was re-streaked on YMA medium supplemented with congo red. To exclude the possibility of growth of colonies from non-perfect sterilization of the nodule surfaces, few microliters from the last sdH_2O solutions used in washing the nodules were streaked on YMA and incubated at 30°C for 5 days along with the nodule extract cultures (Antoun and Prevost, 2006). After the confirmation of perfect sterilizations, pure colonies were kept at 4°C on YMA slants with 1g L^{-1} of $CaCO_3$, and a set of all isolates in broth

media were maintained at - 20°C in glycerol (20%) for further investigations. An example for first isolation and purified plates were shown below (Fig. 3.2).



Sterilized nodules First isolation cultures



A Pure culture A pure culture

Figure 3.2 Nodules, first isolation plates and purified cultures

3.4 Morphological Characterization

3.4.1 Colony Characteristics

To investigate the morphological features of colonies, YMA media supplemented with congo red were used and the colony criteria like diameter, form, color, consistency, production of mucus, opacity…etc were observed (Vincent, 1970).

3.4.2 Gram Stain

One of the confirmatory tests of rhizobia is their negative gram-stain reactions. Thus, they appear as pink rods under microscope after treated with gram stain. For gram stain, a small part of a solid colony or few microliters of the bacterial broth was

spread to a clean glass slide and left for dryness in air or fixed over a slight flame. Then, the slides with fixed bacterial cells (smears) are flooded with crystal violet solution for 1 min. and washed up gently under the tap H_2O. Then iodine solution was applied for 1 min. then rinsed with alcohol for few seconds. Now, the counter stain safranin was applied for 1 min. and then rinsed with H_2O. The smears were then left to air dry and examined under microscope (Somasegaran and Hoben, 1985).

3.5 Acid/alkali Production Test

When inoculated and grown on YMA media supplemented with bromothymol blue (BTB) at pH 6.8, the color changes of the indicator were used to detect the growth rate and alkali/acid production of the isolates as well. The color change of the medium from green to yellow indicated that the isolates were acid-producers and fast-growers whereas the alkali production and the slow growth rate of isolates were concluded when the indicator changed into blue (Mujibar et al., 2000).

3.6 Growth on Glucose Peptone Medium

This is another confirmatory test for rhizobia. When grown on glucose peptone agar (GPA) medium supplemented with bromocresol purple (BCP) as a pH indicator, rhizobia show no or very little growth whereas other contaminating genera like Agrobacteria show abundant growth and cause a change in the pH-indicator color. GPA medium was prepared by dissolving 5 and 10 grams of glucose and peptone, respectively, for each liter of media. GPA was supplemented with 10 ml of BCP stock (1 g in 100 ml ethanol) (Lupwayi and Haque, 1994).

3.7 Keto-lactose Test

This reaction is confirmative for rhizobia. It is the oxidation of lactose in a growth medium to yield a 3-ketolactose. Normally, rhizobia do not have the ketolactase enzyme and thus are not able to utilize lactose from a growth medium containing it. Unlike rhizobia, Agrobacteria genera possess the enzyme lactase and metabolize lactose to produce 3-lactose. The latter can be detected by flooding the cultures with Benedict's reagent which contains Cu ions. 3-ketolacose produced by *Agrobacterium* oxidizes Cu to Cu_2O which precipitate as yellow color (Holt et al., 1994).

3.8 Re-nodulation in Modified Leonard Jars

This assay was used to distinguish between the isolates which were able to re-induce nodule formation and those which were unable to re-nodulate chickpea plants under aseptic conditions in the green house. Seeds of chickpea were surface-disinfected to exclude the possible contamination by nodulating rhizobia adhered to seed surfaces. For this, seeds were soaked in 70% alcohol for 2-3 minutes to get rid of the trapped air. Then, seeds were treated with 3% sodium bleach for 4 minutes and finally rinsed for 5-6 times in sterile distilled water. Afterwards, seeds were left to imbibe in the refrigerator for 1 day or at room temperature for 2-3 hours. After imbibition, seeds were washed for two times in sterile distilled water and transferred by flamed forceps to a growth medium on Petri-dishes (e.g. water agar) or a sterilized combination of vermiculite and perlite. On the growth medium, seeds were incubated at 30°C until seedlings of 0.5-1 cm long were obtained (Woomer et al., 2011). Modified Leonard jars were prepared by cutting ordinary H_2O bottles into 2 halves; one was used as an upper part to contain the growth medium (sterilized vermiculite and perlite, 1:1 w w^{-1}) and the other lower part to hold the irrigation solution of the growing seedlings. A strip of sterilized filter paper was used to convey the irrigation solution to the growing seeds. To keep the roots away from direct light during the growth, the Leonard jars were covered by aluminum foils. Seeds were the planted in the jars and inoculated around the radicles with broth media with sterilized pipettes. The jars were then taken into the green house for incubation at 30°C for 4-5 weeks. N-free solution (Broughton and Dilworth, 1970), with the composition shown in table 3.2, was used to irrigate the seedlings. For plus N-control treatment, potassium nitrate was supplemented to the nutrient solution to a final concentration (0.05%). Three replicates were done for each isolate and a negative control- in which no broth was inoculated to the seedlings- was used. After 35 days, nodule formation is recorded as positive (at least one nodule) or negative (no nodules).

Table 3.2. Nutrient solution (Broughton and Dillworth, 1970)

Stock soln	**Chemical**	**g ml-^{100}**
1	$CaCl_2.2H_2O$	2.94
2	KH_2PO_4	13.61
3	$FeC_6H_5O_7.3H_2O$	0.6700
	$MgSO_4.7H_2O$	12.33
	$K_2SO_4.H_2O$	8.700
	$MnSO_4.H_2O$	0.034
4	H_3PO_3	0.025
	$ZnSO_4.7H_2O$	0.023
	$CuSO_4.5H_2O$	0.010
	$CoSO_4.7H_2O$	0.006
	$Na_2MoO_2.2H_2O$	0.05

Figure 3.3 Renodulation test in modified Leonard jars (A) at the green house (B)

3.9 Nutritional, Physiological and Biochemical Characteristics

For each biochemical and physiological test, inoculation of a loopfull of 48 h old

broth culture was streaked on to the YMA medium. The inoculated YMA Petri-dishes were incubated at 30°C for 2-4 days (Somasegaren and Hoben, 1994). Ultimately, the success of growth for isolates was recorded as (+) for growth and (-) for no growth (Solomon and Fassil, 2014).

3.9.1 Carbohydrate and Amino Acid Utilizations

To test their ability to grow with different substrates as sole sources of carbon, isolates in this study were allowed to grow on carbohydrate-free medium and mannitol, glucose, fructose, galactose, maltose, starch, citrate and sorbitol were used as sole sources of carbon (Somasegaran and Hoben, 1994). To test the growth of the isolates with these carbohydrates, modified YMA medium in which yeast extract was reduced to 0.005 g in 100 ml, was used. The heat stable sugars (glucose, fructose, sucrose, and mannitol) were added, before autoclaving, as 1% (w v^{-1}) to the modified YMA (prepared without mannitol). Similarly, to test the ability of the isolates to utilize different substrates as sole sources of N_2, we grew them with L-alanine, glycine, L-tryptophan, urea, and potassium nitrate as sole nitrogen sources. To test the growth with these N-sources, YMA in which 0.5 g of each of the assessed amino acid replaced yeast extract (Stowers and Eaglesham, 1984). After preparing the basal media with the desired sources of carbon and nitrogen, pH value was adjusted for each medium as 6.8, and then media were autoclaved for 20 min. at 121°C. For each C-and N-source, tests were done in replicates and origin YMA media were used as controls. After 5 days of growth at 30°C, Growth results were recorded upon visual observations as positive or negative (Hungria et al., 2001).

3.9.2 Salt Stress Tolerance

Growth in different salt concentrations were investigated by inoculating YMA medium conatined NaCl at the concentrations 1.0, 2.0, 3.0, 4.0 and 5.0 (w v^{-1}) with suspensions (approximately 10^9 cells ml^{-1}) of cultures. After 7 days of incubation at 30°C, results were recorded qualitatively either as (+) for growth or (-) for no growth (Maatallah et al., 2002) by comparing with the controls which were incubated at 28+2°C (Lupwayl and Haque, 1994).

3.9.3 Maximum Growth Temperature

Growth of bacteria at different temperatures was investigated by inoculating 10 µl

suspension (Approximately 10 cells ml^{-1}) of cultures on YMA plate at 5, 10, 25, 30, 35, 37 and 40°C. The results were recorded qualitatively either as + for growth or - for no growth after seven days of incubation by comparing with control which were incubated at 28+2°C (Hungaria et al., 2000).

3.9.4 Tolerance to Different Acidity and Alkalinity (pH)

Tolerance of the isolates to different acidic and alkaline media were determined on YMA at different pH values of 4, 4.5, 5, 5.5, 6, 8, 8.5, 9, 9.5 and 10 (Gao et al., 1994). 100 ml of YMA medium solution was prepared for each pH test and 0.1N HCl or NaOH were used to set the pH at the desired value. Then 1.5 g agar was mixed with 100 ml YMA solution and autoclaved at 121°C for 15 minutes. YMA plates were prepared with different pH concentration and were then incubated at 30°C for 3 days and growth was visually observed and compared to the growth at the control pH 6.8 (Somasegaran and Hoben, 1994).

3.9.5 Resistance to Antibiotics

To assess their resistances to different antibiotics, various concentrations (µg ml^{-1}) of the following antibiotics were used: Nalidixic acid (NA., 50, 100), streptomycin (Str., 50, 100), kanamycin (KA., 50, 100), erythromycin (Ery., 25, 50), Ampicillin (Amp., 50, 100) and tetracycline (Tetr., 20, 50). After autoclaved, YMA media were supplemented with antibiotics disinfected via filteration using 0.22 µm sized-membrane filters. As described in Lupwayi and Haque (1994). The stock solution of each antibiotic was prepared in 100 ml of water except for erythromycin which was dissolved in ethanol. The required concentration was aseptically added to the media using a single pipette for each antibiotic.

3.9.6 Tolerance to Heavy Metals

To test their ability to tolerate different heavy metals, YMA media with the following heavy metal concentrations (µg ml^{-1}) were used: $CuSO_4$ (Cu, 10, 50, 100), $HgCl_2$ (Hg, 10, 20, 50), $NiCl_2$, $6H_2O$ (Ni, 10, 50, 100), $ZnSO_4$, $7H_2O$ (Zn, 10, 20, 50), $CdCl_2$ (Cd, 5, 10, 20) and $K_2Cr_2O_7$ (Cr, 10, 25, 50). Stock solutions of the heavy metals were disinfected via filterations and supplemented to the media (İçgen et al., 2002).

3.9.7 Biochemical Activities

3.9.7.1 Catalase Enzyme Test

The production of catalase enzyme was determined on YMA media inoculated by the YEM broth of each isolate. After incubation, drops of H_2O_2 were added directly to the colonies or to the colony after being transferred to a glass slide. Air bubbles formation was recorded as positive results.

3.9.7.2 Oxidase Test

The ability of isolates to produce oxidase enzyme was assessed using 1% N, N, N.N-tetramethyle-phenylene diamine. To test for the presence of oxidase, a colony grown on YMA medium was taken and scratched against a strip of filter paper dipped in the reagent and air-dried. The development of purple color indicates positive oxidase.

3.9.7.3 Hydrolysis of Urea

The presence of urease enzyme which helps bacteria hydrolyze urea to liberate nitrate, was tested on YMA supplemented with Urea (2%). Urea was sterilized by filtration and added to the autoclaved YMA medium supplemented with 0.012% phenol red as a pH indicator. After incubation, colonies with urease activitiy turn the indicator to red indicating the alkalinity due to urea hydrolysis and nitrate production.

3.9.7.4 Gelatinase Enzyme Test

YMA media supplemented with gelatin were used to test the ability of each isolate to catalyze gelatin (1%). After growth, cultures were flooded with acidified $HgCl_2$ which precipitate the gelatin into a white precipitate. The transparent halo around the colony meant a positive result and that the isolate could produce gelatinase and catalyze gelatin.

3.9.7.5 Amylase Enzyme Test

The ability of each isolate to produce amylase and catalyze starch was determined on YMA media supplemented with starch (1%). After growth on YMA, cultures were flooded with iodine solution. The transparent halo around the colony meant a positive, while the dark blue precipitate around the colony meant a negative result.

3.9.7.6 Methyl Red (MR) Test

To test their ability to produce stable acids via fermentation of glucose, isolates were inoculated and grown for 4 days in broth media of glucose phosphate peptone. The indicator methyl red was added in few drops and its color change into red indicated the production of acids and positive results.

3.9.7.7 Indole Acetic Acid (IAA) Production Test

When grown on a growth medium with tryptophan, some microorganisms are able to metabolize tryptophan via tryptophanases enzyme to produce indole acetic acid. The production of acid lowers the pH value of the growth medium which can be detected through a color change of a suitable indicator. Isolates in this study were grown in tryptone broth media and Kovac's reagent was used as the indicator. When indole is produced, it reacts with 4 (p)-dimethylaminobenzaldehyde contained in the indicator and a violet red color was observed and taken as positive indole result.

3.10 Numerical Analysis of Phenotypic Variables

The final matrix contained 133 isolates (120 nodulating and 13 NEB endophytes) and 71 traits. Results were recorded 1 and 0 for positive and negative, respectively, and hierarchical clustering analysis was carried out using IBM SPSS version 23 software. Pearson correlation interval measure and between-groups linkage method were used for the hierarchical clustering.

3.11 Molecular Characteristics

3.11.1 DNA Extraction and Integrity

DNA Extraction were done by Purelink Genomic DNA Mini Kit (Code: K182002) according to the manufacture instructions as follows: YEM broth (3 ml) was centrifuged in a sterile 1.5 ml microcentrifuge tube for 4 min at 12000 rpm. The digestion buffer was used (180 µl) to resuspend the pellet and 20 µl proteinase K was added to break the cells down. After mixing, the tubes were incubated at 55°C with continuous vortexing. To get rid of RNA in the sample, 20 µl RNA was added and tubes were left at room temperature for 2 minutes, and then centrifuged for 5 minutes at room temperature. In a new tube, 200 µl lysis/binding buffer was mixed with the supernatant, blended well via vortexing and then left at room temperature for 120

sec. To precipitate the DNA from the lysate, 200 µl absolute ethanol was added and vortexed gently for 30 seconds. The lysate was then transferred to a spin column tube and centrifuged at room temperature at 10,000 × g for 60 sec. The column was placed into a new collection tube along with 500 µl wash buffer 1 and centrifuged at 10,000 × g for 1 minute at room temperature. The spin column was transferred to a new collection tube along with 500 µl of wash buffer 2, then centrifuged at maximum for 4 min. The spin column was then transferred to a new Eppendorf tube and the DNA is eluted from the spin column in 100 µl sterile distilled water or elution buffer by centrifugation at maximum speed for 1 minute. Now, the Eppendorf contained the gDNA and the spin column is discarded or used for another elution of an extra DNA. DNA can be kept at 4°C for frequent use or -20°C for long-term use.

After gDNA was obtained, its intactness and integrity can be checked for via 1% agarose gel electrophoresis with 1X TAE and ethidium bromide (1.5 µg ml^{-1}). DNA was prepared by mixing 1 µl gDNA with 3 µl 1X TE buffer (pH 8) and 1 µl 5X loading dye. Samples were then vortexed to maintain homogeneity and gel was run for 1hr. at ~120 volt in 1X TAE buffer. Genomic DNA samples were left to migrate a distance of at least 1 cm distant from the wells in the gel and to allow a clear vision of the marker bands. Gel was then removed and imaged. Genomic DNA should appear as a single band with no smearing.

3.11.2 Rep-PCR Reactions

The repetitive extragenic palindromic (REP) DNA sequences for the isolates were examined by using primers (REP IR1 and REP 2-I) and The REP-PCR reaction (de Bruijn, 1992) comprised the following ingredients; 1 µl of 200-250 ng of the gDNA, 0.5 µl (final conc. is 50 picomol) of each of the primers; the forward REP IR-1 (5'-IIIICGICGICATCIGGC-3') and the reverse REP 2-I (5'-ICGICTTATCIGGCCTAC-3'), 0.4 µl Taq (from the stock tube (5 u μl^{-1}), 2.5 DMSO (from a 99.5% stock), 3.14 µl dNTPs (from an aliquot of 10 mM), 7 µl $MgCL_2$, 2.5 µl polymerase Taq buffer, and the total volume is made up to 25 µl with sterilized M.Q H_2O. The temperature cycles (De Bruijn, 1992) used for amplifying the REP-units were: 1 initial denaturation cycle at 95°C for 6 min, 30 cycles at 94°C (denaturation) for 1 min, at 40°C for 1 min (annealing), and at 65°C (extension) for 8 min; 1 final extension cycle at 65°C for 14 min; and a final soak at 4°C. The REP-PCR products, along

with a molecular marker, were separated onto 1% agarose gel with ethidium bromide for 2 hours at 75 volts. To each 4 µl PCR reaction products, 1 µl loading buffer (prepared by mixing 2.5 ml of 1% bromophenol with 4 g sucrose and M.Q was added up to 10 ml) was added before injection in the gel. When the dye reaches the end of the gel, the gels were photographed by using a Polaroid film.

3.11.3 Amplification and Analysis of 16S rDNA Genes of NRE Isolates

After the REP-PCR reaction was performed to amplify the characteristic band patterns of REP-elements, representative isolates were selected (at random) for sequencing their 16S rRNA genes to determine their identity. For this, the bacterial DNA extracted with the commercial kit as described before, was used for a PCR run for amplifying the 16S rRNA genes. The template DNA (1 µl of 100-150 ng) of each representative isolate was used per reaction in the amplification reaction which comprised the following ingredients; using 1.0 µl from an aliquot of 0.2 µM of the forward 41f (5'-GCTCAGATTGAACGCTGGCG-3') and the reverse primer 1488r (5'-CGGTTACCTTGTTACGACTTCACC-3'), 200 µM dNTPs and 0.2 µl from 5 u μl^{-1} stock Taq enzyme and complete the reaction volume up to volume 25 µl with sterilized M.Q. H_2O.

PCR Amplifications were performed in a PCR thermocycler and the temperature profile cycles were as follows; 5 min. at 95°C (initial denaturation) 1 cycle, 30 cycles at 94°C (denaturation) for 60 sec., at 72°C for 60 sec. (annealing), and at 72°C (extension) for 7 min; for final extension 1 cycle at 72°C for 7 min; and a final hold at 4°C. After PCR, electrophoresis was run using 5 µl of PCR products in 0.7% agarose gels at 75 V until the colorant reaches the end of the gel. Molecular size marker of 1 kb DNA was used to compare the obtained band sizes. The characteristic band of the 16S rRNA was checked at about 1400-1500 nucleotides base pairs.

PCR purified products were prepared for sequencing reactions and analyzed in a DNA sequencer with dye primers. Sequences of the whole representative isolates along with those of the type strains were put together and were prepared for obtaining phylogenetic trees to know the exact position of each isolate with respect to the type strains. For this purpose, computer-assisted DNA and protein sequence analyses were performed using NCBI (http://www.ncbi.nlm.nih.gov) network servers. After applied to the BLAST sequencing at NCBI, the identity of each

representative isolates were determined through the highest similarity percentage of the nearest strain identified in the database. To draw a phylogenetic tree, the sequences of the representative isolates along with sequences of strains to which highest similarity records and the gram-positive Bacillus were used.

CHAPTER IV
RESULTS

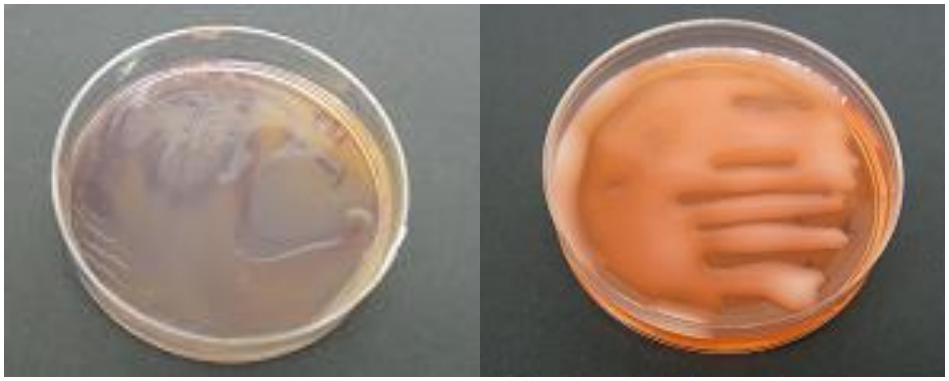
4.1 Isolation of Root-nodule Bacteria

In this research study, root-nodules of chickpea collected from 17 cities in six provinces in Turkey, were used as the source for isolating a total of 120 root-nodulating rhizobia (RNR) and 13 non-nodulating endophytic bacteria (NEB). YMA media supplemented with cong red were used as the selective media for isolation.

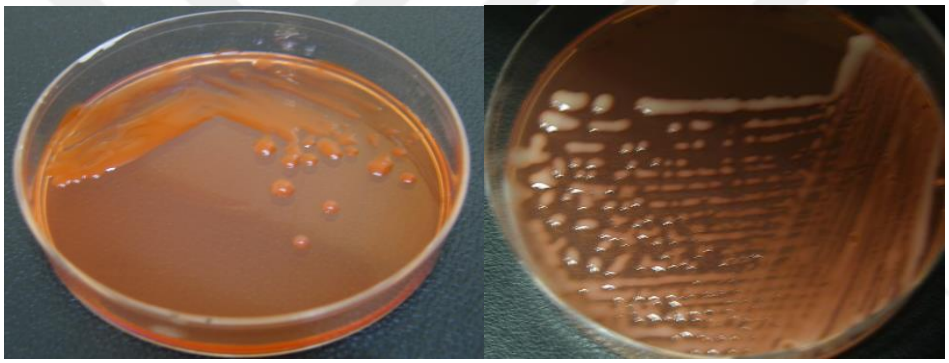
4.2 Colony Characteristics

Based on colony morphology, isolates in the nodulating group (RNR) showed 2 main morphotypes; 90% of the isolates in this group produced mucous, circular, smooth-margined watery colonies with 2-4 mm diameter after 1-3 days of growth at 28°C on YMA medium. The second phenotype, showed by 10% of the isolates in the RNR group, was creamy, non-mucous, opaque colonies with larger colony diameter (3-5 mm) (Fig. 4.2 and 4.3). None of the isolates in both morphotypes absorbed CR on YMA and all of them were gram-negative rods. In addition, for the RNR group, only 9 isolates (Kon9, Kir19, Kir4, Hat4A, Gsenlik1, Cor2A, Adi15, Adi17 and Ada8) showed moderate growth on GPA and 9 isolates (Kony7, Kir19, Gsut2, Gsehinb, Esk7B, Cor4, Aks3A, Afy1 and Adi1) showed positive ketolactose. In the second group of NEB isolates, it displayed also two main morphotypes; one morphotype with yellowish white colonies (2 isolates) and the other with light pink colonies (11 isolates). The isolates in this non-nodulating group were all gram-negative rods under microscope. All the isolates in this later (NEB) group grew on GPA medium except for two isolates (Cor5NEB and Kir13NEB) while 6 isolates (Afy5NEB, Cor5NEB, Cor7NEB, Kah1NEB, Mers10NEB and Kah3NEB) were positive to ketolactose test. According to their growth rates on YMA media, based on the growth rate on the standard medium, all the isolates in both groups (RNR and NEB) were found to be fast growers giving the acidic BTB reaction result (Fig. 4.13). While the data obtained from phenotypic characterizations are considered inadequate

for precise determination of the identity of isolates, they are helpful for intentatively evaluating the isolates diversity.

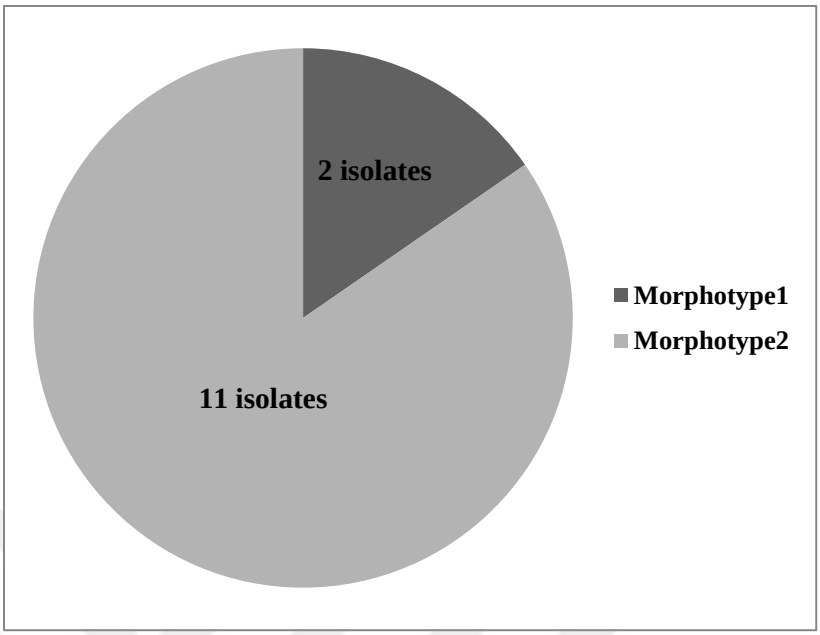


NEB morphotype 1 **RNR morphotype 1**

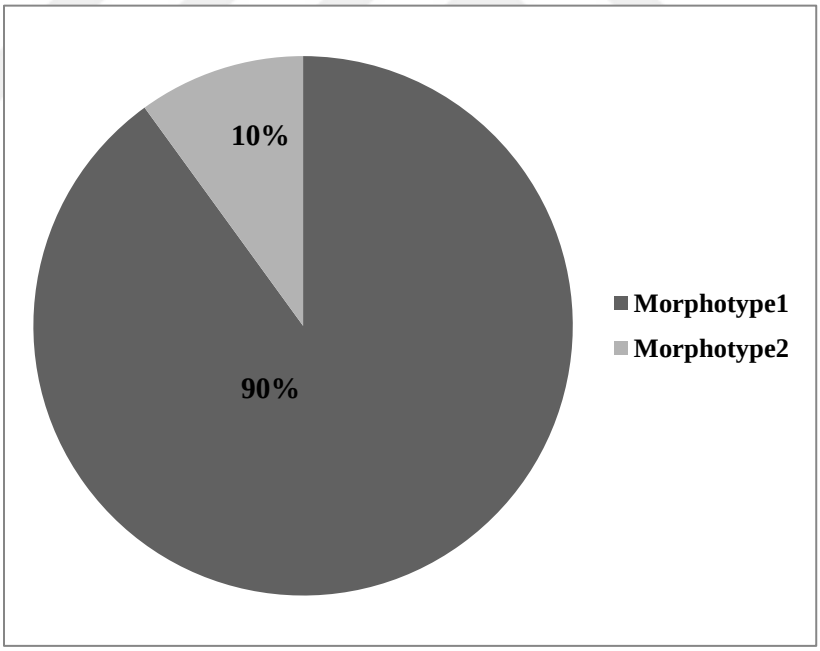


NEB morphotype 2 **RNR morphotype 2**

Figure 4.1 Morphology of colonies of isolates from RNR and NEB groups from chickpea nodules in Turkish soils



NEB Morphotype 1: Slightly pink mucous colonies
NEB Morphotype 2: Yellow to yellowish white watery colonies



RNR Morphotype 1: mucously, circular, smooth-margined and watery colonies
RNR Morphotype 2 creamy, non-mucously, opaque colonies

Figure 4.2 Morphotypes of NEB and RNR isolates

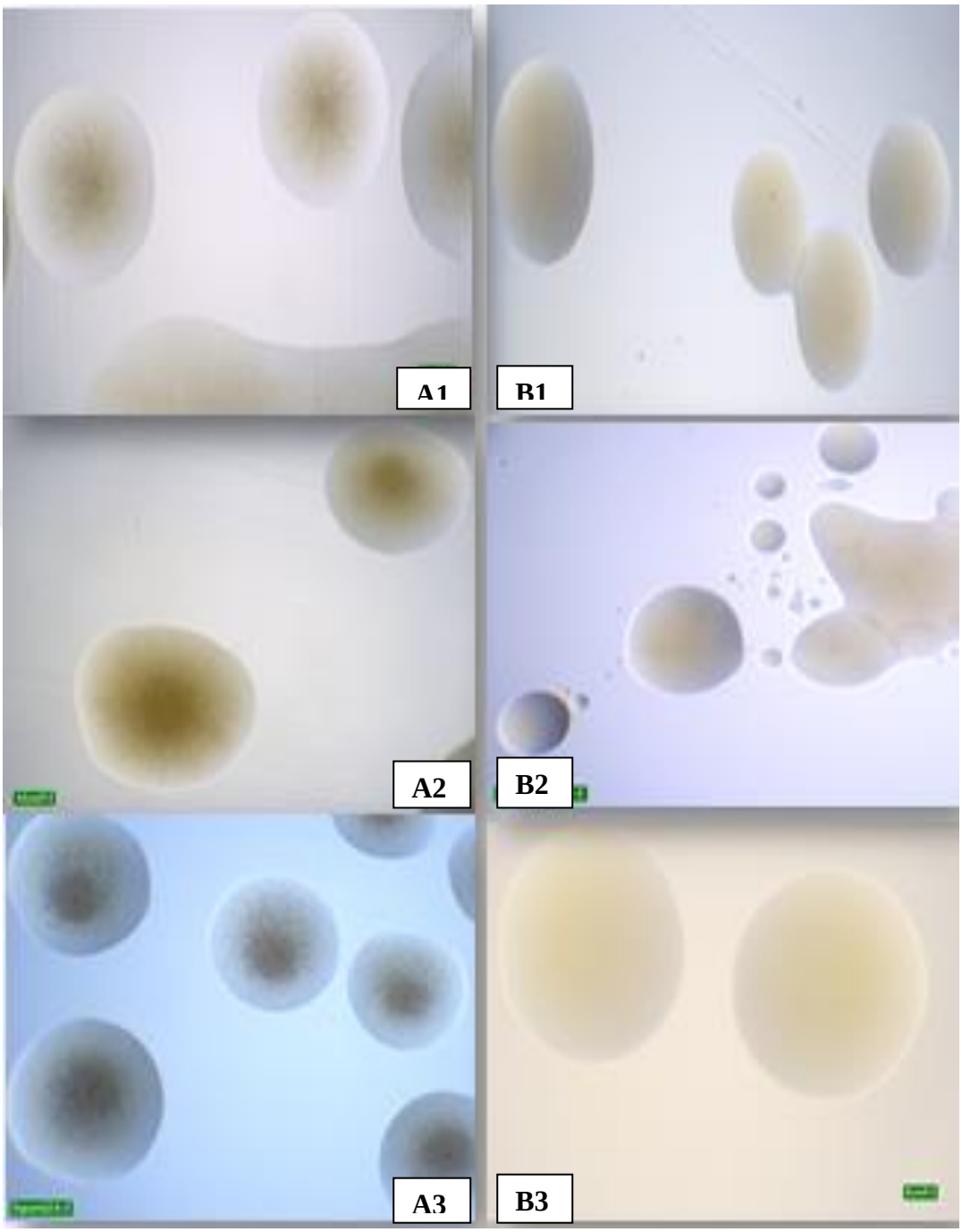


Figure 4.3 Colonies of RNR (A1, A2, andA3) and RNR (B1, B2 and B3) isolates under microscope

4.3 Re-nodulation Tests

In the nodulation assay, a single nodule on the root system indicated positive while absence of nodules ment negative nodulability. 120 were found to be able to nodulate

their host plants and they were termed root-nodulating rhizobia RNR while 13 isolates were found to be non-nodulating endophytes (NEB) (Table 4.1).

Table 4.1. Nodulating and non-nodulating isolates in this study

Groups	Isolates within the group
RNR isolates	Ada2/Ada5A/Ada6A/Ada8/Ada9/Ada10/Adi1/Adi4/Adi5/Adi6/Adi7/Adi9/Adi12A/Adi14/Adi15/Adi16A1/Adi17Afy1/Afy3/Afy5Aks1/Aks2/Aks3A/Aks3B/Aks4A/Aks5/Aks7/Aks10Amas3/Amas4/Amas7/Cor2A/Cor3/Cor4/Cor5/Cor7/Cor9/Esk6/Esk7A/Esk7/BGbak/Gbur1A/Gbur1B/Gbur3/Gogu1/Gogu2/Gsar1A/Gsehinb/Gsehit1Gsenlik1/Gsut2/Gyav1/Gyav3A/Gyes1Hat1/Hat2/Hat3/Hat4A/Kah1/Kah2A/Kah3/Kah6/Kah7/Kah8/Kah9/Kah10/Kah13/Kir1/Kir4/Kir5A/Kir9/Kir10/Kir12/Kir15/Kir17A/Kir19/Kir20/Kir21/Kon2/Kon3/Kon7/Kon9/Kut2/Kut9/Mer1/Mer2/Mer3/Mer4/Mer5A/Mer5B/Mer6A/Mer6B/Mer7/Mer8/Mer9/Mer10A/Mer11/Mer12/Mer13/Mer16/Mer17/Mer18/Mer26/Tok1/Tok2/Tok3/Tok4/Urfa2/Usak1/Usak2/Usak13/Usak17/Yoz2/Yoz6/Yoz7/Yoz8/Yoz11/Yoz14/Yoz15/Yoz16
NEB isolates	Ad2NEB/Ad5NEB/Afy1NEB/Afy5NEB/Cor5NEB/Cor7NEB/Kah1NEB/Kah3NEB/Kir11NEB/Kir12NEB/Kir13NEB/Kut9NEB/Mers10NEB

For codes of isolates see table 3.1

Figure 4.4 Positive nodulation of some isolates of chickpea in this study

4.4 Physiological Characterizations of Isolates of This Study

4.4.1 Carbon and Nitrogen Utilizations

Concerning carbon and nitrogen source utilizations (Table A1.1 and Fig. 4.5), the nodulating isolates (RNR) displayed the ability to utilize numerous substrates as sole sources of carbon. They all with no exception utilized mannitol, glucose, fructose, galactose, and maltose, while few isolates (29.1%) metabolized starch and 16% and

33.1% utilized sorbitol and citrate, respectively. Similarly, isolates of this group showed diversity in utilizing different N-sources. They all grew with yeast extract, alanine and glycine with no exception, while they respond variably to tryptophan, urea and sodium nitrate. With respect to the non-nodulating NEB isolates (Table A.1.6 and Fig. 4.6), they showed similar responses to the nodulating group as they all grew with mannitol, glucose, fructose, galactose, and maltose, but showed lesser response to starch, citrate and sorbitol. The isolates in this latter group were all able to utilize yeast extract, alanine and glycine, while showed the least utilization in case of sodium nitrate (Only 4 isolates out of 13).

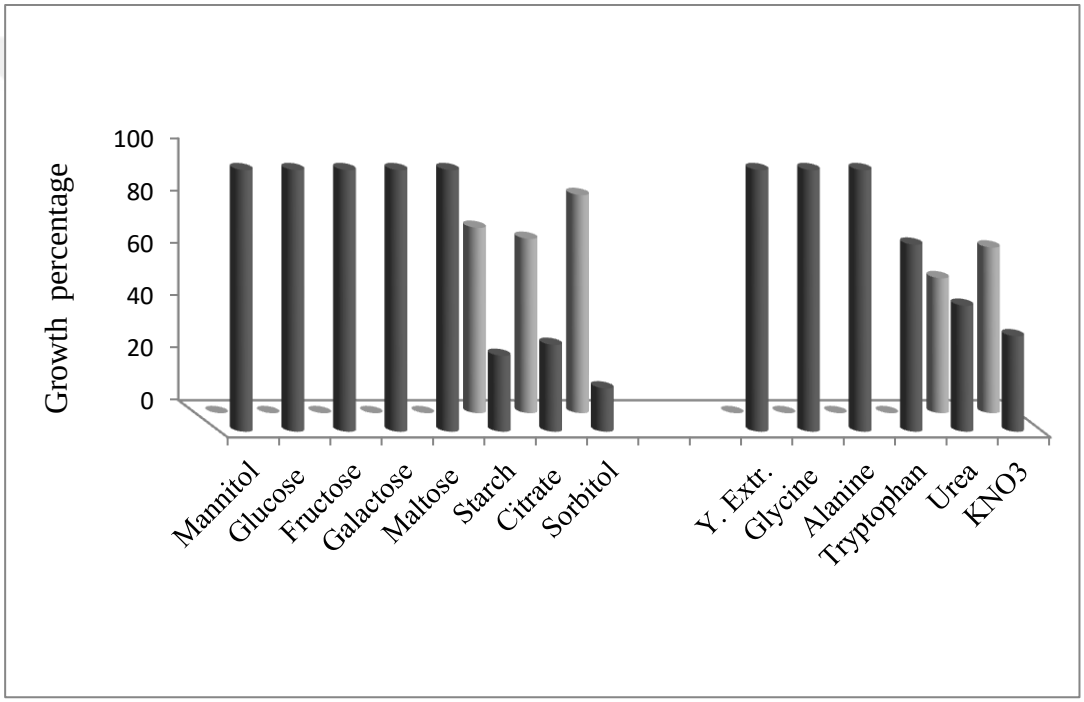


Figure 4.5 Growth of RNR isolates with C and N sources. Black columns indicate growth while grey indicate no growth percentage of isolates

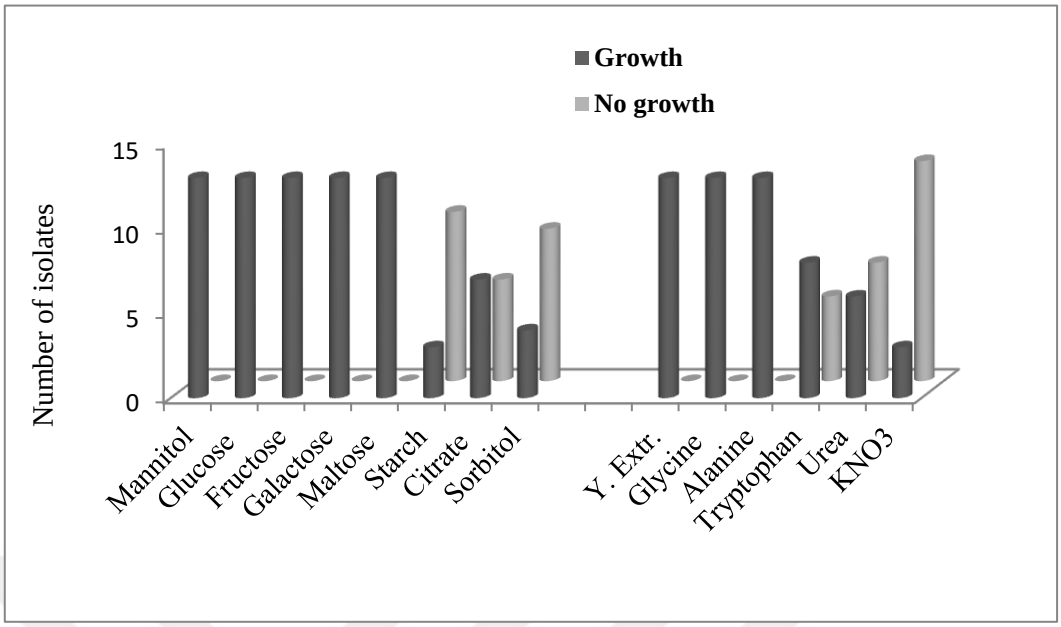


Figure 4.6 Growth of NEB isolates with different C and N sources.

4.4.2 Salt, Temperature and pH Tolerance

Isolates in this study showed different tolerance to salinity (NaCl) stress. 100% of the nodulating isolates (120) tolerated low concentrations of NaCl (1 and 2%) and at higher concentrations, number of tolerant isolates decreased as salinity level increases. 10 isolates (8.3%) viz. Ada10, Adi4, Adi12A, Adi16A, Amas7, Cor7, Tok1 and Tok4, Esk7B and Afy5 endured 5% NaCl (Table A.1.2 and Fig. 4.7). With respect to the non-nodulating bacteria (NEB), they showed thermotolerant features as they displayed higher responses to salinity; they were all able to withstand up to 4% NaCl. Even at 5% NaCl, 11 isolates were able to grow. Salinity tolerance was not dependent on the collection site as variations in salt tolerance were observed in the isolates from the same collection site in both groups.

Isolates in this study showed similar variation with respect to growth at different temperatures. The optimum temperatures for growth for the RNR group was between 20 and 35°C as the majority of the isolates was able to grow at 20-35°C. Below and above these temperatures, growth decreased obviously. However, some isolates showed thermotolerant features as they had optimum temperature between 35-45°C. Moreover, some isolates were able to grow at 15°C (36 isolates) and 45°C (13 isolates) and none of the isolates of this group endured beyond 45°C (Table 4.2). For

NEB, the isolates showed optimum growth between 15 and 35°C. Interestingly, some isolates (Ad5NEB, Afy1NEB, Afy5NEB, Cor7NEB and Kut9NEB) of this group grew at 50°C, and none of them endured above 50°C (Table 4.3).

For pH stress tolerance in this study, interestingly, isolates in the nodulating group showed growth at both acidic and alkaline pH values as 51.6% and 62.5% survived at pH 5 and pH 9, respectively. However, 10% survived at alkaline pH 10 (Table 4.2 and A1.3). The optimum pH for the majority of isolates in this group was between pH 6 and pH 8. With respect to NEB group, the isolates in this group showed a wider range or tolerance to pH values and alkalotolerant features as they were all able to grow between 6-10 pH (Table A.1.7 and Fig. 4.8).

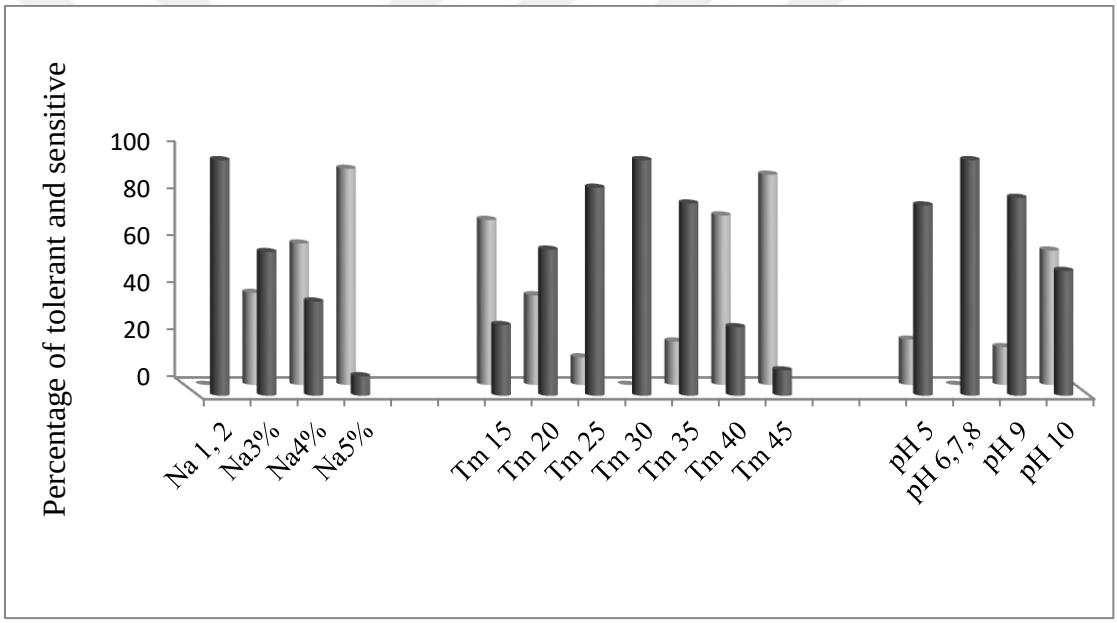


Figure 4.7 Percentage of tolerant isolates of the nodulating group at different values of salinity (Na), temperature (Tm) and pH. Different values of the same variable type that gave the same result were combined under one column. Dark columns indicate tolerant while light indicate sensitive

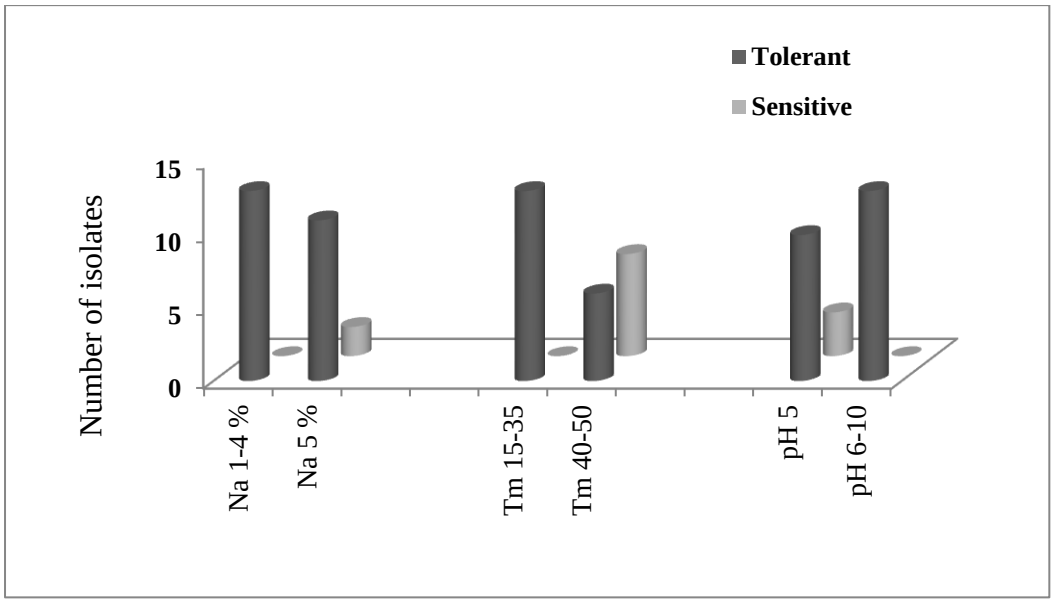


Figure 4.8 Tolerant isolates of the non-nodulating group at different values of salinity (Na), temperature (Tm) and pH. Different values of the same variable type that gave the same result were combined under one column.

4.4.3 Antibiotic Resistance and Heavy Metal Tolerance

Assessment of antibiotic stress tolerance, of our isolates in the nodulating group, showed the high tolerance against nalidixic acid (100% and 88.3% endured 50 and 100 µg ml^{-1}, respectively), streptomycin (100% tolerated 50 µg ml^{-1} and 75% tolerated 100 µg), kanamycin (80.8% and 70.8% survived at 50 and 100 µg ml^{-1}, respectively) and tetracycline (100% survived at 20 and 67.5% at 50 µg ml^{-1}). Meanwhile, erythromycin and ampicillin were more effective against the isolates of this group. The RNR isolates were insensitive to most of the assessed antibiotics (Fig. 4.9). The highest AB tolerance was in the order, nalidixix acid > streptomycin> kanamycin > tetracycline > ampicillin > erythromycin. Isolates in the NEB (Fig. 4.10) showed similar insensitivity to most of the tested concentrations of the evaluated antibiotics; all the 13 isolates resisted low concentrations (µg ml^{-1}) of nalidix acid (50), tetracycline (20) and streptomycin (50), and more than 75% of the isolates in this group grew with the highest dozes of streptomycin (100) and nalidix acid (100). With respect to heavy metal stress, isolates in both groups also showed variable degrees of tolerance to the tested metals (Tables A.1.3 and A.1.8). At the lower concentrations of the tested metals, high tolerances were recorded in case of RNR isolates to Hg (all the 120 isolates survived at 10 µg ml^{-1}), Cr (88.3% grew at 10 µg ml^{-1}) and Cu (81.6% grew at 10 µg ml^{-1}). This degree of tolerance decreased

with increasing the dozes of the metals. Also, isolates of the NEB group showed a higher degree of tolerance (than RNR isolates) to the majority of the evaluated heavy metals at different concentrations; all the isolates in this group tolerated the lower concentrations of the whole metals with no exception (Fig. 3.13). The highest tolerance in this group were recorded to Zn, Cd and Cr. Cu and Ni was lethal to thee isolates in this group at highest concentrations.

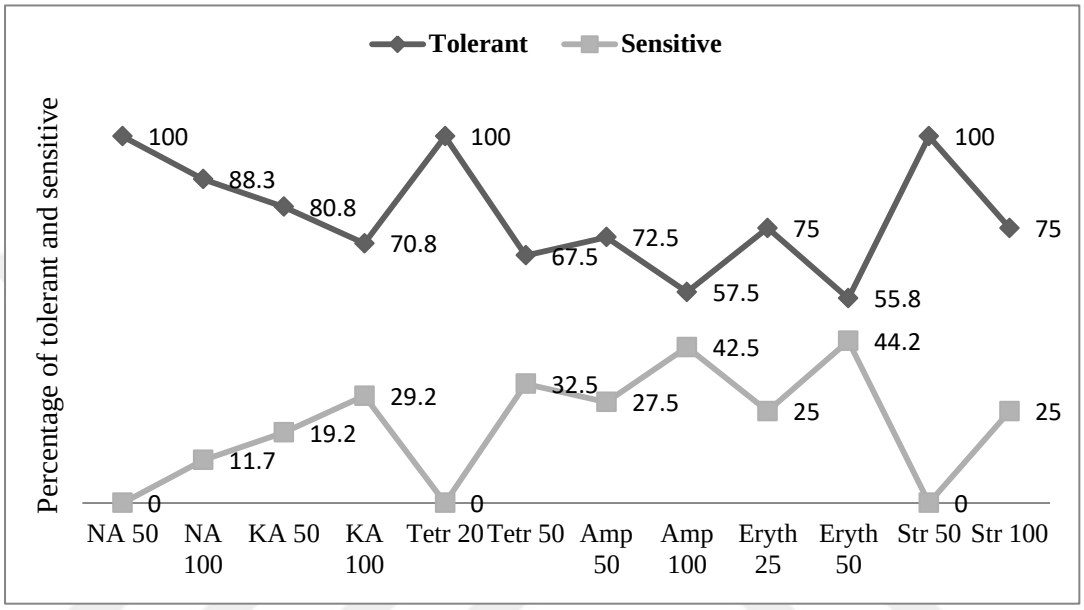


Figure 4.9 Percentage of tolerant and sensitive isolates in RNR group at different concentrations (µg ml^{-1}) of the tested antibiotics

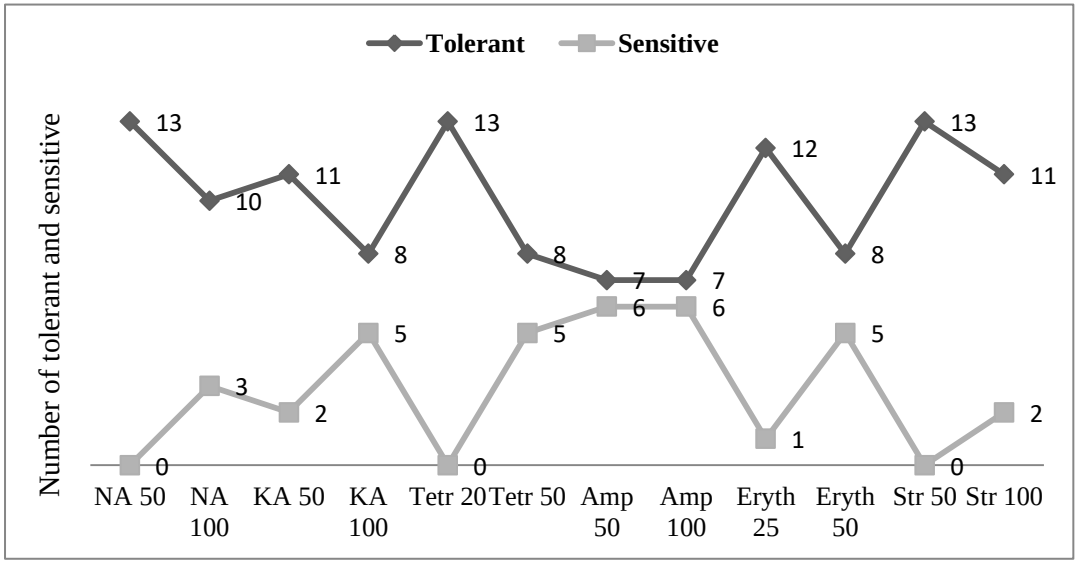


Figure 4.10 Number of tolerant and sensitive isolates in NEB group at different concentrations (µg ml^{-1}) of the tested antibiotics

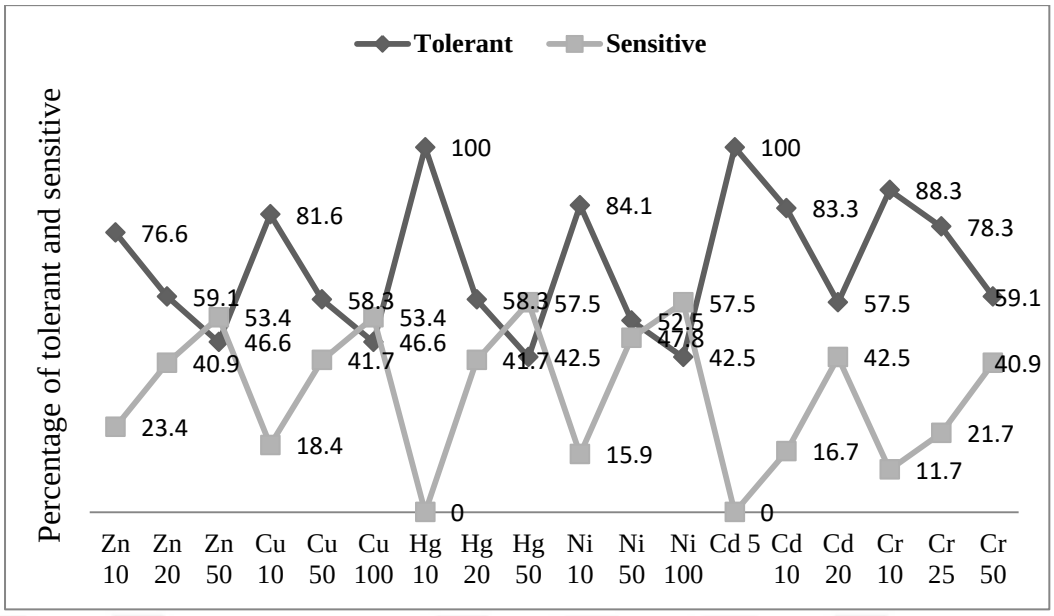


Figure 4.11 Percentage of tolerant and sensitive isolates in RNR at different concentrations (µg ml^{-1}) of heavy metals

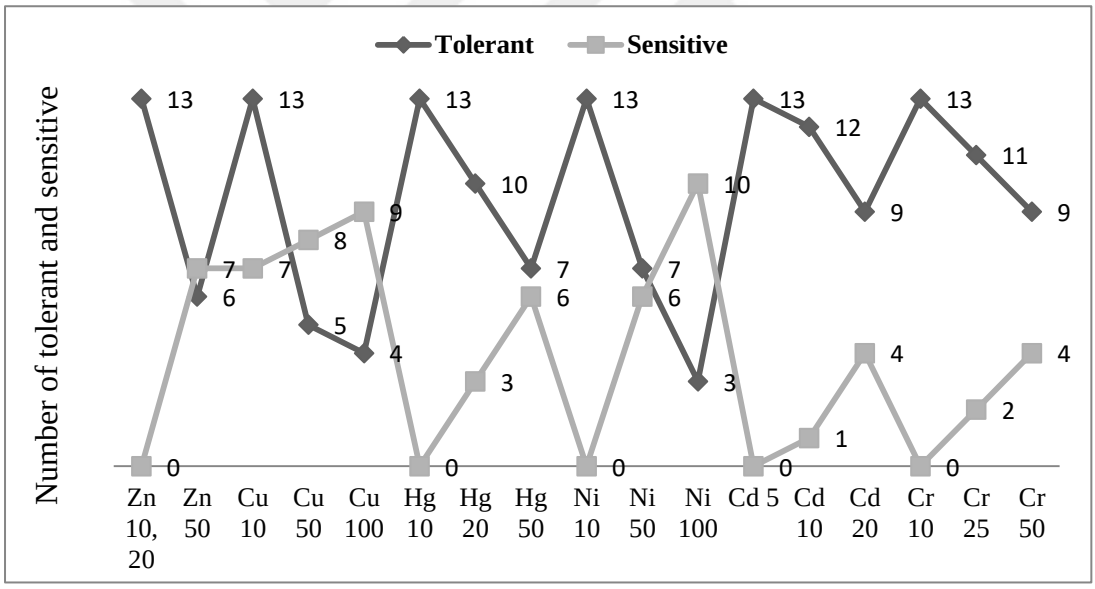


Figure 4.12 Number of tolerant and sensitive isolates of NEB isolates at different concentrations (µg ml^{-1}) of heavy metals

4.4.4 Biochemical Activities

In this study, isolates in the RNR group showed variable biochemical activities; they were all catalase and oxidase positive, many of them were positive to urea (72 isolates) and some were positive to indolee acetic acid (18 isolates). No positive activity was recorded for gelatinase, MR or amylase. For the NEB isolates, they were

all catalase, oxidase and gelatinase positive, and some of them showed positive ketolactose (6 isolates) and urease (7 isolates) tests.

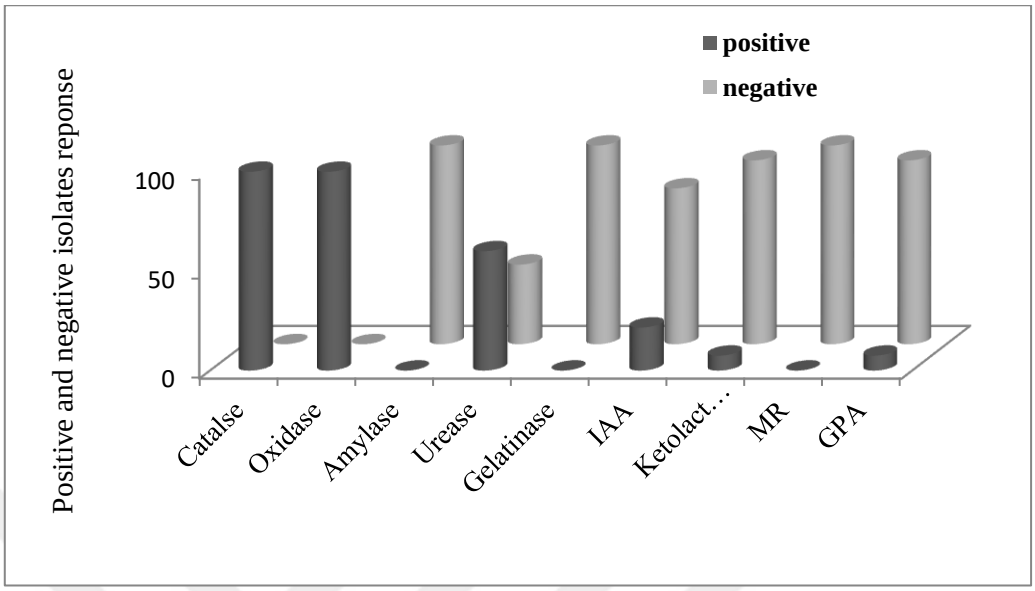


Figure 4.13 Biochemical activities of RNR isolates

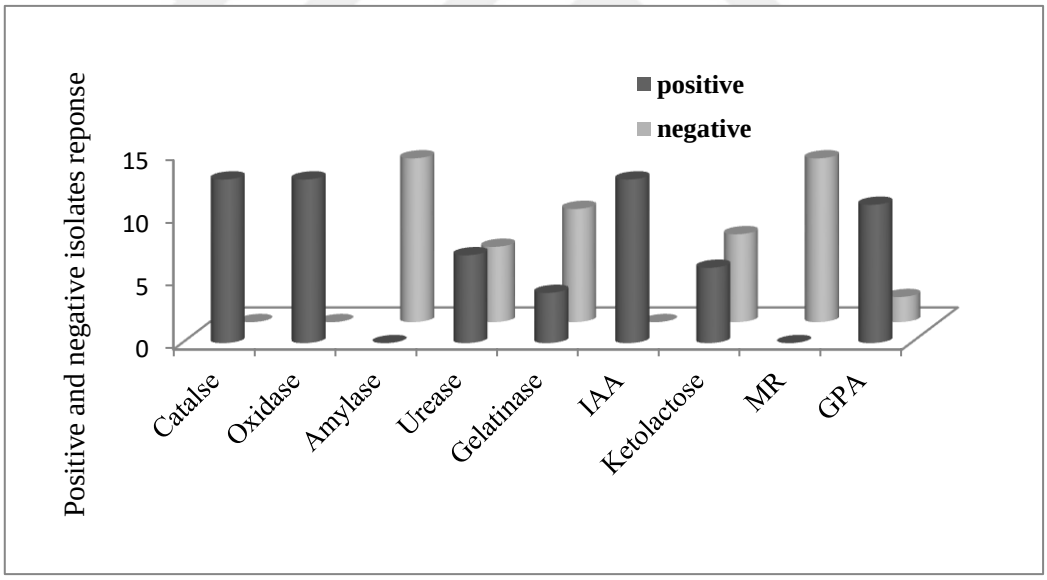
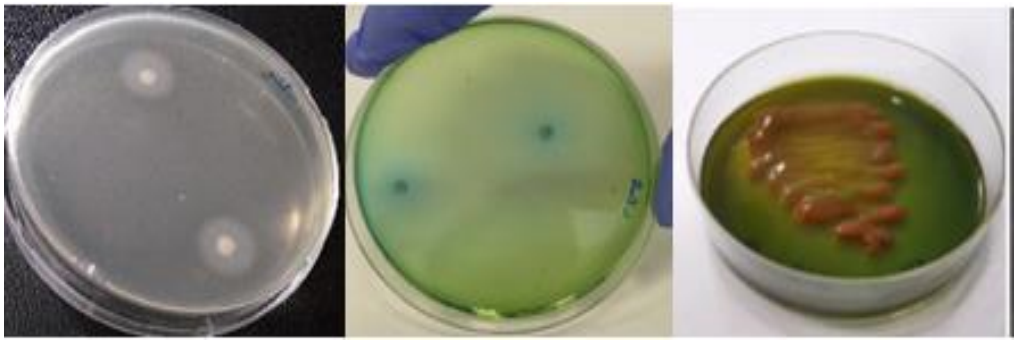
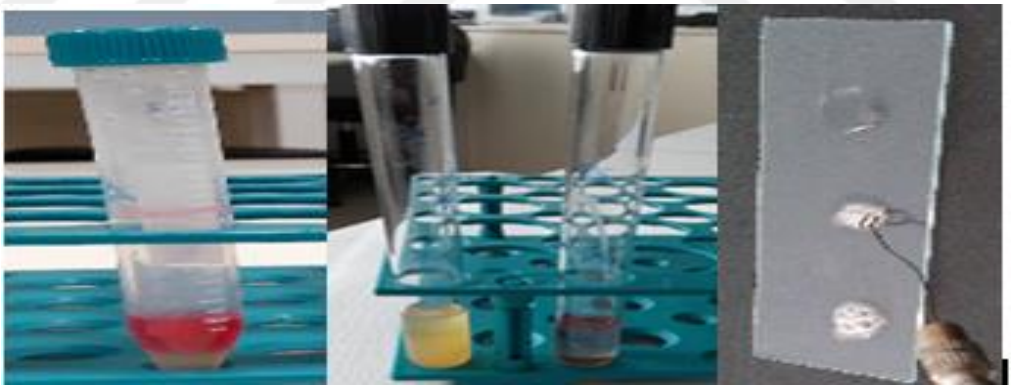


Figure 4.14 Biochemical activities of NEB isolates



Positive gelatinase Positive citrate Positive MR

Negative amylase Positive Urease positive Oxidase



Positive MR positive IAA positive catalase

Figure 4.15 Some biochemical activities of isolates in this study

4.4.5 Cluster Analysis

The final matrix contained 120 isolates in the RNR and 13 in the NEB group, and 71 phenotypic traits for both groups. Results' codes were 1 and 0 for positive and negative, respectively. Hierarchical clustering analysis was carried out using IBM SPSS version 23 software. Pearson correlation interval measure and between-groups

linkage method were used for the hierarchical clustering. Cluster analysis placed isolates of RNR group into 3 clusters at 25% similarity (Fig. 4.16). Cluster I had 113 isolates. They came from different origins and metabolized different carbon and nitrogen compounds. They moderately tolerated 1 to 3% NaCl and pHs between 5 and 10 and were sensitive to temperatures above 35°C. They showed high tolerance to Cr, Cd, Zn, Ni, NA, and KA and were IAA and urease positive. Cluster I isolates showed a close relationship with *Mesorhizobium cicer*. Cluster 2 had 5 isolates viz. Adi1, Afy1, Esk7B, Gsehinb, and Kir19. They displayed higher tolerance to salinity and temperature than isolates in cluster I as they all grew at 3% Na and between 35-45°C. They also tolerated all concentrations of Zn, tetracycline, and streptomycin and were all urease positive. Based on these traits and descriptions in previous approaches, they displayed close phenotypic similarities to *Mesorhizobium mediterraneum*. Two isolates namely Adi15 and Afyon5 fell into cluster 3. Unlike other isolates, cluster 3 isolates did not utilize starch as a sole carbon source but grew well with sorbitol and citrate and endured salinity up to 4%. They all grew between 20-40°C and withstand all concentrations of Ni, Cd, NA, and streptomycin, but were sensitive to Ampicillin. Upon their traits and feautures described by Jarvis et al. (1982) and Nour et al. (1994, 1995), they were tentatively related to *Mesorhizobium* sp. clustering of isolates did not correlate with their collection sites. Atypical example of this was isolates Afy1 and Kir19 which came from diverse sites but clustered together in cluster 2. For NEB isolates, they were classified into 3 clusters. (fig. 4.17 and table 4.3). Cluster 1 came with 4 isolates viz. Ada2NEB, Kah1NEB, Kah3NEB and Kir13NEB. All the isolates in this cluster were halotolerant (tolerated salinity up to 5% NaCl), were all insensitive to all the tested concentrations of the antibiotic streptomycin (up to 100 µg ml^{-1}) and the heavy metals Cr (up to 50 µg ml^{-1}), and NA (up to 100 µg ml^{-1}). Also, cluster 1 isolates were all urease negative and sensitive to moderate and high concentrations of Cu (50 and 100 µg ml^{-1}). Cluster 2 had 4 isolates, to wit, Ada5NEB, Afy1NEB, Afy5NEB and Kut9NEB. These isolates were similar to those of the previous cluster in their salinity tolerance, as they all withstood NaCl up to 5%). Also, 3 isolates of this cluster were tolerant to 50°C. However, none of them was able to utilize tryptophan, urea or nitrate as a sole source of nitrogen. Besides, all the isolates in this cluster were sensitive to Ni (50 and 100 µg ml^{-1}) and to a lesser degree to Cu; 1 of 4 isolates was able to grow at 50 and 100 (µg ml^{-1}). Also, one isolate in this cluster was able to utilize each of sorbitol, starch

and citrate. Cluster 3 comprised 5 isolates namely, Cor5NEB Cor7NEB Kir11NEB Kir12NEB and Mers10NEB. The isolates in this cluster were all ketolactose positive. They were all also insensitive to KA and Hg, but tolerant to Amp at all their tested concentrations.

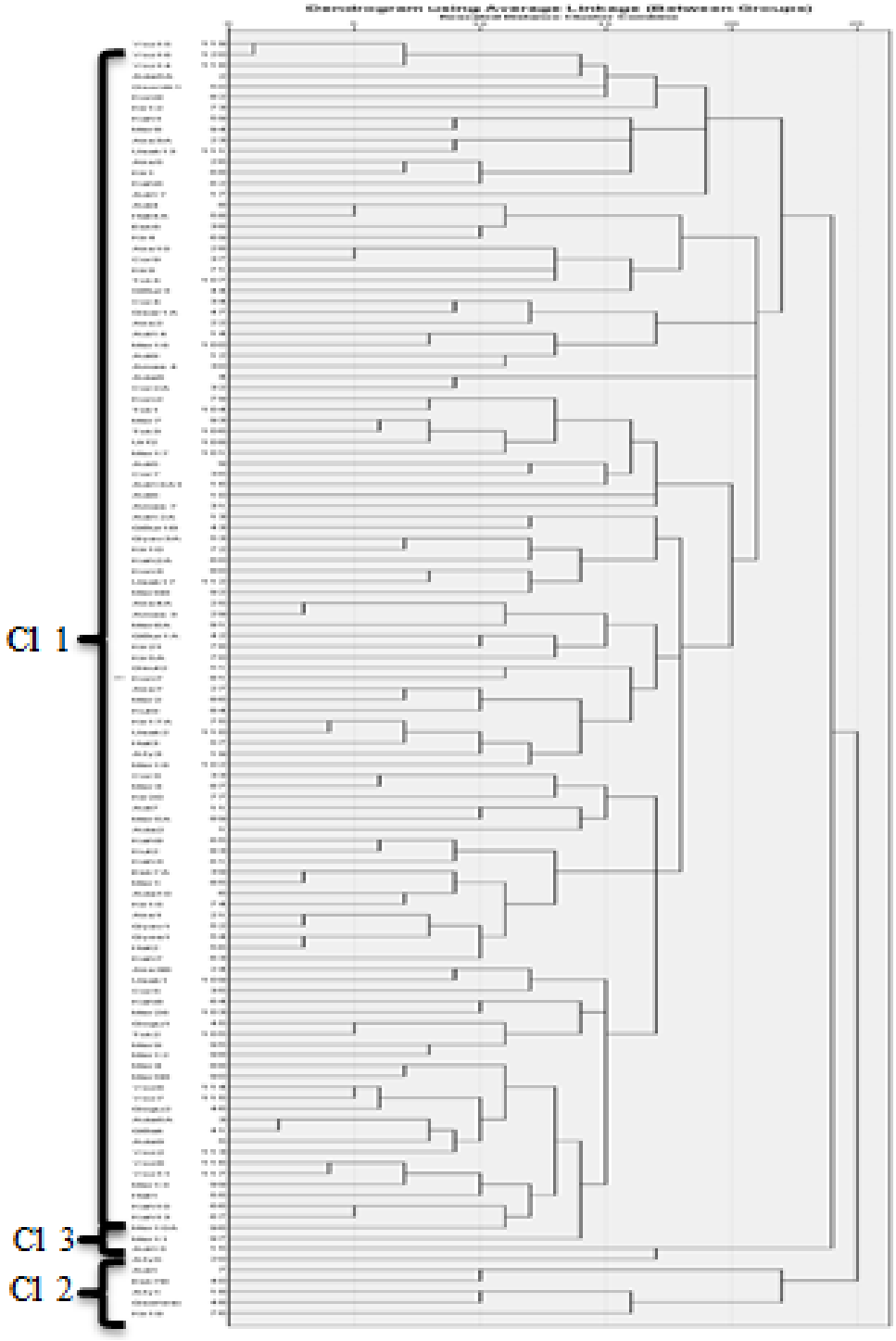


Figure 4.16 Phenogram showing clusters (Cl) of 120 chickpea-nodulating isolates (RNR) from different areas of Turkey

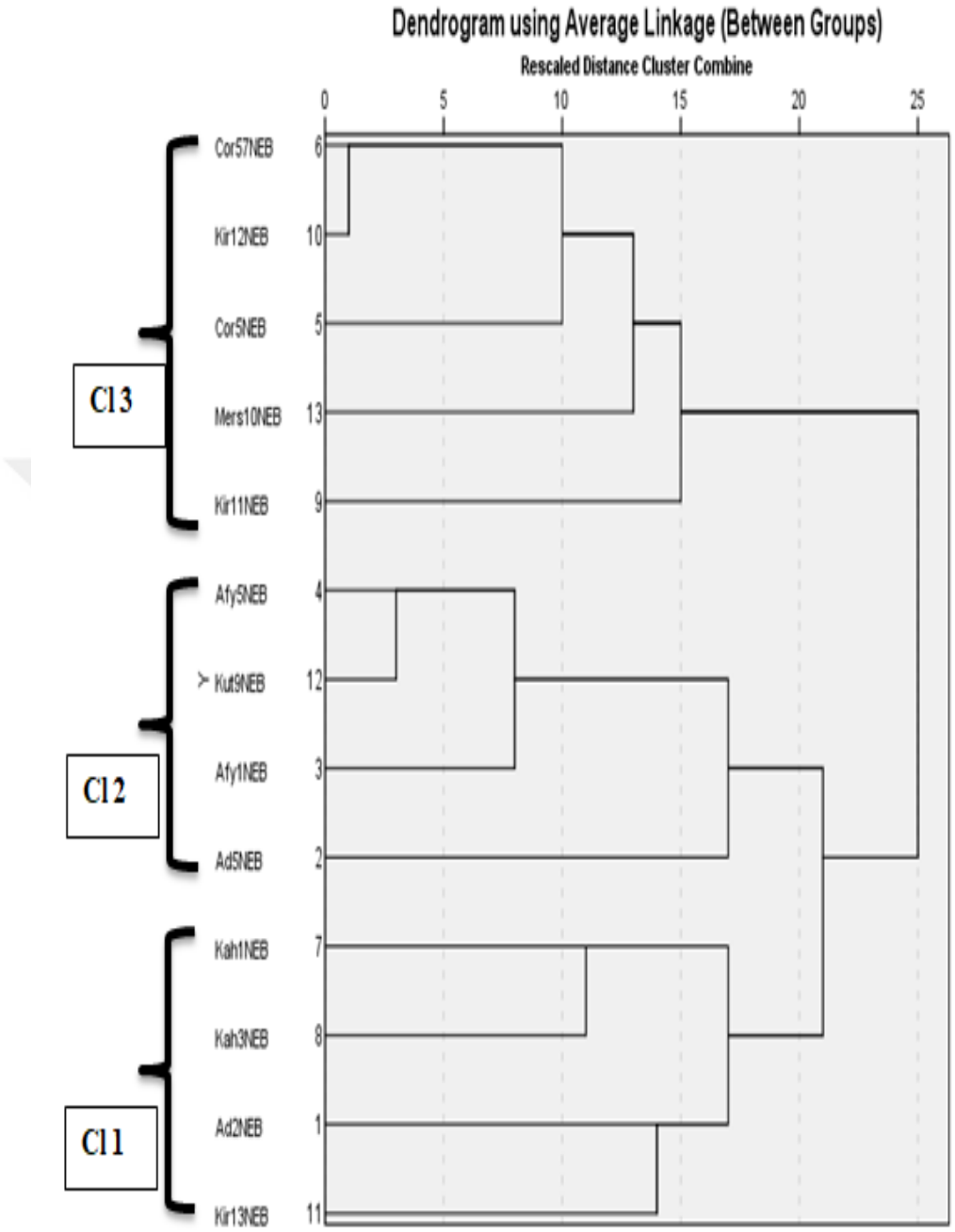


Figure 4.17 Phenogram showing clusters (Cl) of 13 chickpea nodule endophytic (non-nodulating) bacterial isolates (NEB) from different areas of Turkey

Table 4.2 Clusters in each group of RNR and NEB isolates based on phenotypic characterizations

Clusters	Isolates in the cluster
Clus 1 RNR	Ada2/Ada5A/Ada6A/Ada8/Ada9/Ada10 /Adi4/Adi5/Adi6/Adi7/ Adi9/Adi12A/Adi14/Adi16A1/ Adi17 /Afy3/Aks1/Aks2/Aks3A/ Aks3B/Aks4A/Aks5/Aks7/Aks10Amas3/Amas4/Amas7/Cor2A/Cor3/ Cor4/Cor5/Cor7/Cor9/Esk6/Esk7A/Gbak/Gbur1A/Gbur1B/Gbur3/ Gogu1/Gogu2/Gsar1A/ /Gsehit1/Gsenlik1/Gsut2/Gyav1/Gyav3A/ Gyes1Hat1/Hat2/Hat3/ Hat4A/Kah1/Kah2A/Kah3/Kah6/Kah7/Kah8/ Kah9/Kah10/Kah13/Kir1/Kir4/Kir5A/Kir9/Kir10/Kir12/Kir15/Kir1A/ Kir20/Kir21/Kon2/Kon3/Kon7/Kon9/Kut2/Kut9/Mer1/Mer2/Mer3/ Mer4/Mer5A/Mer5B/Mer6A/Mer6B/Mer7/Mer8/Mer9/Mer10A/Mer1 1/Mer12/Mer13/Mer16/Mer17/Mer18/Mer26/Tok1/Tok2/Tok3/Tok4/ Urfa2/Usak1/Usak2/Usak13/Usak17/Yoz2/Yoz6/Yoz7/Yoz8/Yoz11/ Yoz14/Yoz15/Yoz16
Clus 2 RNR	Adi1/Afy1/Esk7B/Gsehinb/Kir19
Clus 3 RNR	Adi15/Afy5
Clus 1 NEB	Ada2NEB/Kah1NEB/Kah3NEB /Kir13NEB
Clus 2 NER	Ada5NEB/Afy1NEB/Afy5NEB/Kut9NEB
Clus 3 NEB	Cor5NEB Cor7NEB/Kir11NEB/Kir12NEB/Mers10NEB

Table 4.3 Clustering of phenotypic traits of 120 isolates nodulating chickpea

Characteristics	**Cluster1 (*n=113)**	**Cluster2 (n=5)**	**Cluster3 (n=2)**
Cofirmation tests			
GPA, Ketolactose	4,7	5,1	0,1
Carbon utilization			
Starch, Sorbitol, Citrate	33,22,46	2,2,2	0,2,2
Nitrogen utilization			
Tryptophan, Urea, KNO3	83,56,41	2,1,2	1,1,1
Salinity tolerance			
3, 4, 5%	67,43,8	5,3,1	2,2,1
Temperature Tolerance			
15, 20, 25, 35, 40, 45^{o}C	34,81,102,91,28,8	1,2,2,5,5,5	1,2,2,2,2,0
pH tolerance			
5, 9, 10	92,100,61	4,3,3	1,2,0
Heavy metal resistance			
Zn 10, 20, 50	86,66,51	5,5,5	1,0,0
Cu 10, 50, 100	94,68,55	2,1,1	2,1,0
Hg 20, 50	64,47	4,2	2,1
Ni 10, 50, 100	94,58,47	5,3,2	2,2,2
Cd 10, 20	97,67	1,0	2,2
Cr 10, 25, 50	109,89,67	5,4,3	2,1,1
Antibiotic resistance			
NA 100	102	2	2
KA 50, 100	92,82	4,3	1,0
Tetr 50	75	5	1
Amp 50, 100	84,66	3,3	0,0
Eryth 25, 50	86,66	2,0	2,1
Strept 100	83	5	2
Enzyme activities			
Urease	67	5	0
IAA	23	1	2

* N denotes isolates number per cluster and column numbers are the isolates that gave a positive reaction. Commas (,) were used to separate different values of the same trait and their corresponding responses in clusters in a respective series. Phenotypes that gave the same results in all strains were omitted from the table.

Table 4.4 Clustering of phenotypic traits of 13 isolates unable to nodulate chickpea

Characteristics	**Clus1 (*n=4)**	**Clus2 (n=4)**	**Clus3 (n=5)**
Cofirmation tests			
GPA, Ketolactose	2,2	1,4	3,5
Carbon utilization			
Starch, Citrate, Sorbitol	2,3,2	1,1,1	2,3,1
Nitrogen utilization			
Tryptophan, Urea, KNO_3	3,2,1	0,0,0	5,4,2
Salinity tolerance			
3, 4 and 5%	4,4,4	4,4,4	5,4,3
Temperature Tolerance			
40, 45 and 50°C	2,1,1	4,4,3	2,2,1
pH tolerance			
pH 5	3	3	4
Heavy metal resistance ($\mu g\ ml^{-1}$)			
Zn 50	1	3	2
Cu 50, 100	0,0	1,1	4,3
Hg 20, 50	2,1	3,1	5,5
Ni 50, 100	3,3	0,0	4,0
Cd 10, 20	4,3	3,3	5,3
Cr 25, 50	4,4	2,2	5,3
Antibiotic resistance ($\mu g\ ml^{-1}$)			
NA 50, 100	4,4	4,2	5,4
KA 50, 100	3,2	3,1	5,5
Tetr 50	3	3	2
Amp 50, 100	3,3	4,4	0,0
Eryth 25, 50	4,3	3,2	5,3
Strept 100	4	4	3
Enzyme activities			
Urease	0	3	4
Gelatinase	1	2	1

***** N represents isolates number per cluster and column numbers are the isolates giving a positive reaction. Commas (,) were used to separate different values of the same trait and their corresponding responses in clusters in a respective series. Phenotypes that gave the same results in all strains were omitted from the table.

4.4.6 Molecular Characterization

4.4.6.1 DNA Extraction

After genomic DNA extractions were done with the purelink genomic DNA mini kit (Code: K182002) upon the instructions of the manufacturer, DNA was tested for integrity through electrophoresis. The quality of patterns obtained from DNA migrations indicated that DNA of the selected isolates was clean and not degraded. The DNA obtained via kit in this study varied between 1000-3000 ng ml^{-1}. A characteristic band of the genomic DNA was obtained for each isolate.

4.4.6.2 Rep-PCR Patterns of Root-nodulating Bacteria

Through amplifications of REP-elements via PCR, very clear and discriminative patterns were obtained. These patterns could be used for distinguishing species pattern differences between bacterial isolates. This technique was used as the molecular method for displaying the diversity patterns of 120 isolates in the RNR and 13 isolates of the NEB group from chickpea nodules. To amplify the characteristic patterns of REP-units, a set of the 5 pmol of the forward REP IR-1 (5'-IIIICGICGICATCIGGC-3') and the reverse REP 2-I (5'-ICGICTTATCIGGCCTAC-3'), (De Bruijn, 1992) was utilized for the REP-units amplifications which are present in numerous repetitions along the DNA of the invistigated isolates. Results (Fig. 4.18 to 4.20) from the fingerprinting resolvable band patterns of the consensus sequences of REP-units indicated that isolates had a wide range of diversity. As it was proposed (Versalovic et al., 1991), REP elements constituted a useful tool for fingerprinting bacterial genomes.

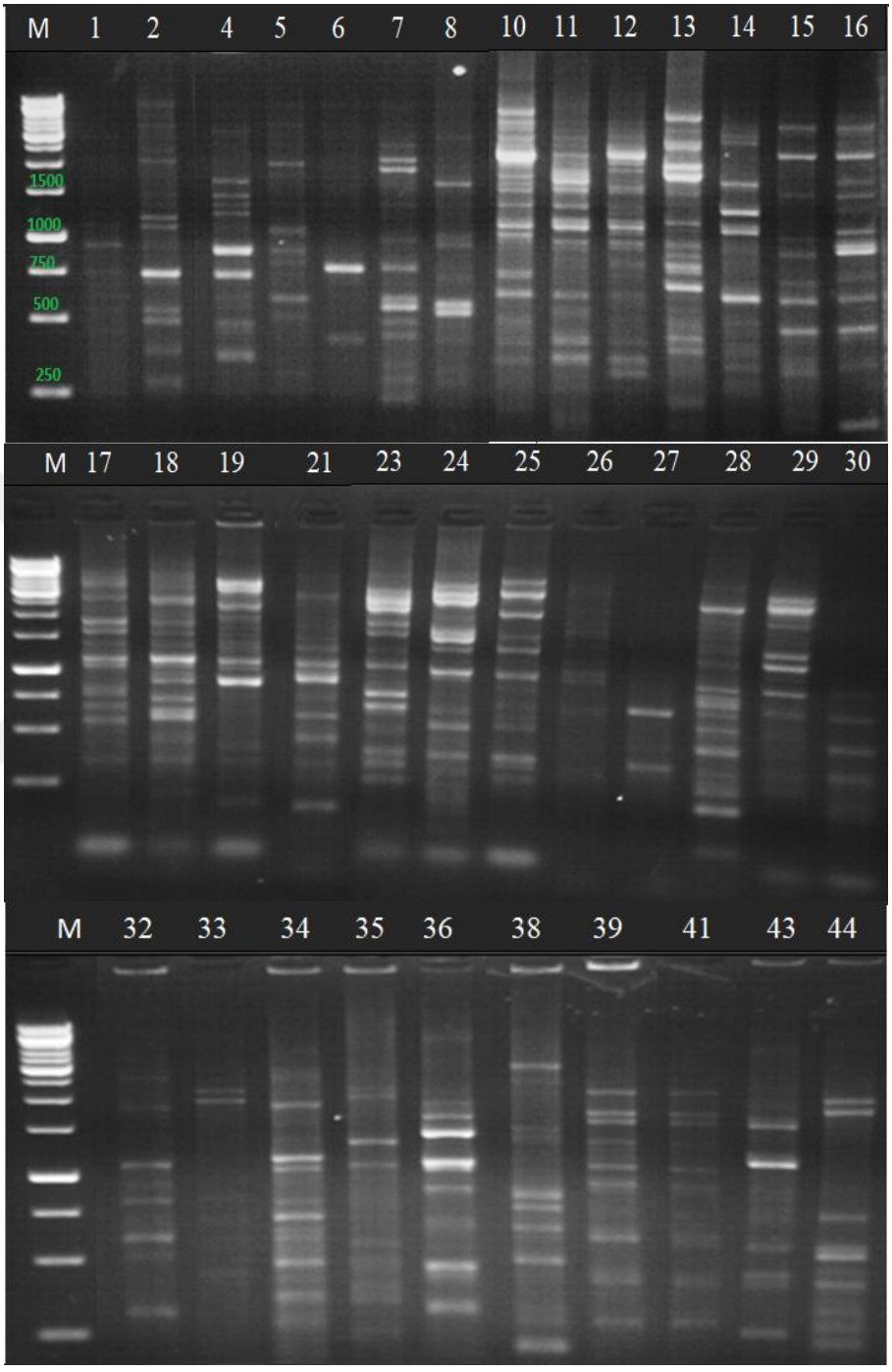


Figure 4.18 REP-PCR fingerprint patterns generated by using the REP primers. Lanes shows the REP PCR pattern of chromosomal DNA of RNR group

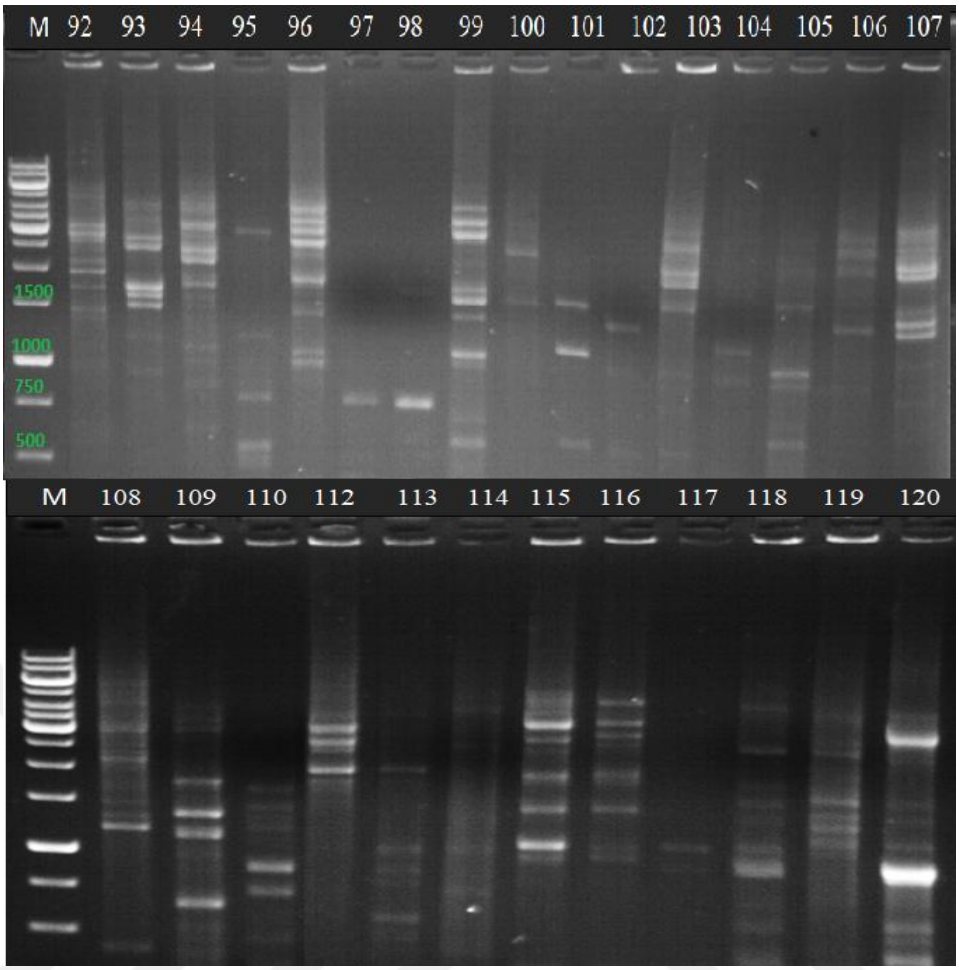


Figure 4.19 REP-PCR fingerprint patterns generated by using the REP primers. Lanes shows the REP PCR pattern of chromosomal DNA of RNR group

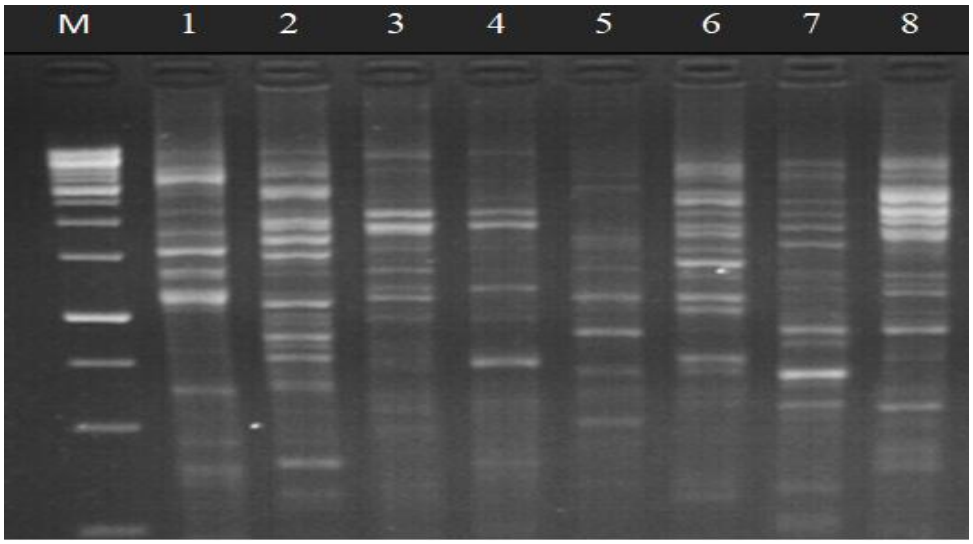


Figure 4.20 REP-PCR fingerprint patterns generated by using the REP primers. Lanes shows the REP PCR pattern of chromosomal DNA of of NEB strains Afy1NEB, Afy5NEB, Cor5NEB, Cor7NEB, Kah1NEB, Kah3NEB, Kir12NEB, Kir13NEB, Kut9NEB and Mers10NEB were loaded in lanes 1 to 8, respectively

4.4.6.3 16s rDNA Amplifications and Phylogenetic Tree

To determine the accurate identity of the isolate, the 16S rRNA genes' sequences were amplified and then compared to the previously identified sequences in the databases to determine the highest percentages of sequence similarity. For this, the forward 41f (5'-GCTCAGATTGAACGCTGGCG-3') and the reverse 1488r (5'-CGGTTACCTTGTTACGACTTCACC-3') primers were used, 16S rDNA gene was amplified for 8 representative isolates of the NEB group viz. NEB-Afy5, NEB-Cor5, NEB-Kah7, NEB-Kir12, NEB-Kir20, NEB-Kut9 and NEB-Mers10. The 16S rDNA characteristic bands appeared at 1400-1500 bp in agarsoe gels (Fig. 4.21). The alignment sequences for the 8 representative strains were done to determine the precise taxonomic position of each isolate to the sequences of the already identified strains in the databases and choose the type strains to which the isolates recorded the highest percentage of similarity. Results in table (Table 4.4) showed the representative isolates and their identity according to the highest similarities of their 16S rRNA gene sequences to the sequences of standard strains from the databank. The identified strains fell within 4 genera with sequence similarity 99%. NEB-Ad5 was identified as *Rahnella aquatilis*, NEB-Cor5 as *Pseudomonas koreensis*, NEB-Kah7 as *Rhizobium nepotum*. 5 isolates were identified within *Enterobacter cloacae* viz.NEB-Afy5, NEB-Kir12, NEB-Kir20, NEB-Kut9 and NEB-Mers10. To draw a phylogentic tree for the the 8 representative isolates of the NEB group, their sequences along with the sequences of the standard strains to which these representatives recorded the highst similarity sequence percent, were combined and aligned using MEGA version 7.0 (Fig. 4.22) and a standard Bacillus sequence was added to get a rooted-phylogenetic tree. Results showed that out of 8 sequenced isolates 5 isolates identified within the genus *Enterobacter.*

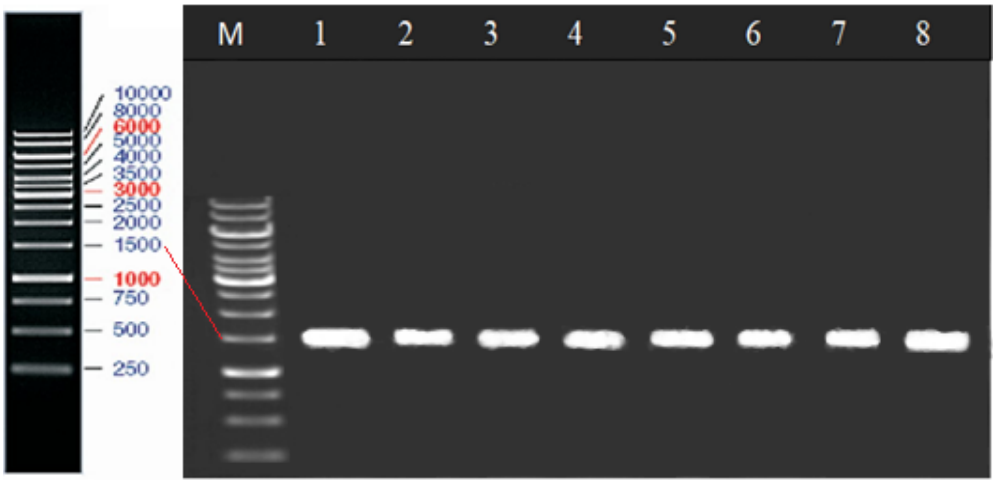


Figure 4.21 The 16S rRNA genes amplifications of 8 representative isolates; NEBAd5, NEBAfy5, NEBCor5, NEBKah7, NEBKir12, NEBKir20, NEBKut9 and NEBMers10 (lanes 1 to 8, respectively), the bands were separated on A 0.7% agarose gel using the primers 14f and 1488r.

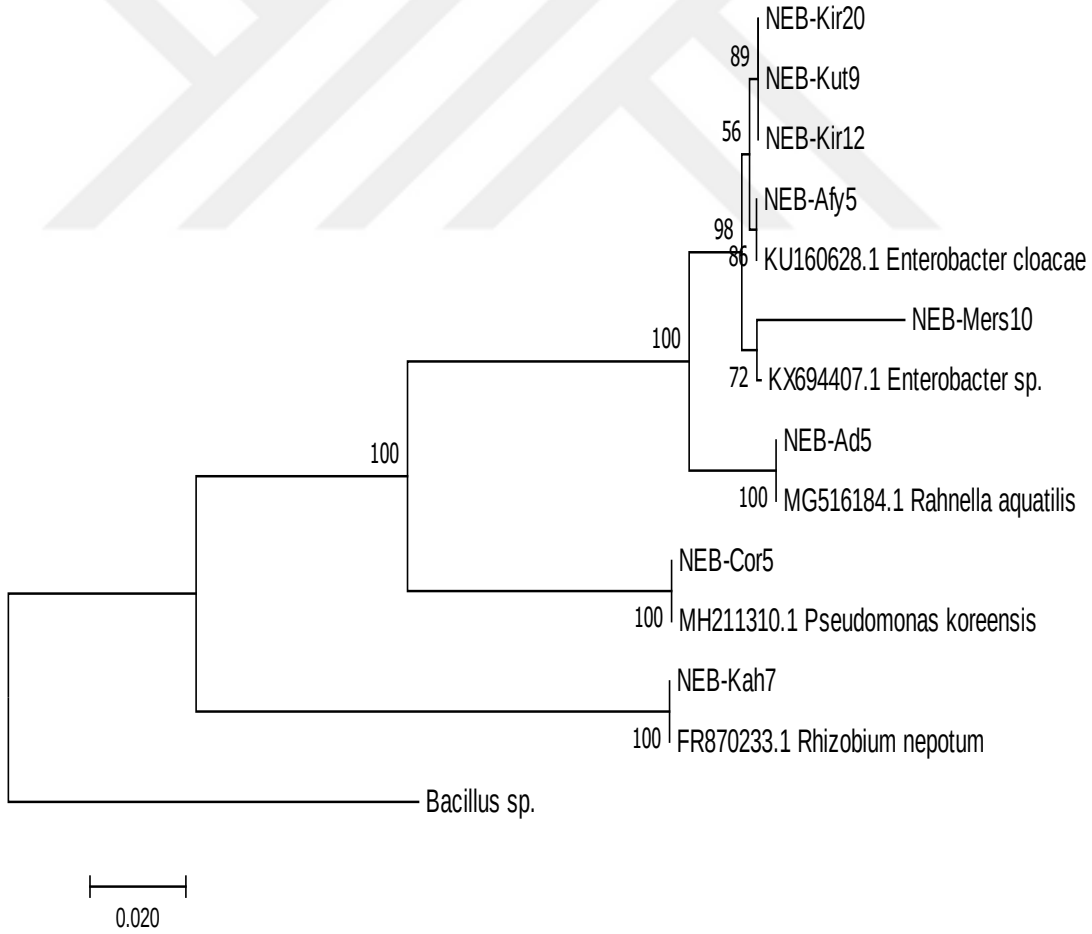


Figure 4.22 Phylogenetic tree based on aligned sequence of 16S rDNA. Bootstrap probabilities are indicated at the branching points

The Neighbor-joining method was used to infer the evolutionary history. The optimal tree with the sum of branch length = 0.45087523 is shown. Next to the branches was shown the percentage of replicate trees in which the associated taxa clustered together in the 1000 replicates' bootstrap test. The distances of evolution were computed using the Kimura 2-parameter method and were in the units of the number of base substitutions per site. The analysis involved 14 nucleotide sequences. Codon positions included were $1^{st}+2^{nd}+3^{rd}$+Noncoding. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 613 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

Table 4.5 16S rRNA identification of 8 representative isolates in the NEB group

Isolate	99% Similarity with	Accession number
NEB-Ad5	*Rahnella aquatilis*	SUB40460124 MH349001
NEB-Afy5	*Enterobacter cloacae*	SUB404601226 MH349002
NEB-Cor5	*Pseudomonas koreensis*	SUB404601245 MH349003
NEB-Kah7	*Rhizobium nepotum*	SUB404601284 MH349004
NEB-Kir12	*Enterobacter cloacae*	SUB4046012 95 MH349005
NEB-Kir20	*Enterobacter cloacae*	SUB4046012100 MH349006
NEB-Kut9	*Enterobacter cloacae*	SUB4046012 107 MH349007
NEB-Mers10	*Enterobacter* sp.	SUB4046012 123 MH349008

CHAPTER V

DISCUSSION

Phenotypic and molecular characterizations of root-nodule bacteria of chickpea (*Cicer arietinum*) from different growing regions in Turkey have revealed a wide range of diversity. This heterogeneity in response to the physiological and biochemical stress parameters, in the populations of chickpea root-nodule bacteria, was reported in several previous studies, whose findings were in line with or sometimes contradicted our findings, as explained in the few lines to come.

5.1 Morphology and Re-nodulability of Chickpea Root-nodule Bacteria

In this study, growth and cultural characteristics of nodulating root-nodule isolates (RNR) were in line with the general characteristics of fast-growing rhizobia described in many bacteriology manuals (Jordan 1984). Most of our RNR isolates produced mucous and formed circular, smooth-margined and watery colonies with medium diamters (2-4 mm) after one to three days of incubation on YMA medium supplemented with congo red. The colony characteristics (Fig. 4.1) of our isolates coincided with the previous findings by Jida and Assefa (2012). Similar morphological characteristics including colony morphology, pigmentation and consistency were the basis to study chickpea-nodulating bacteria (Gauri et al., 2012; Rai et al., 2013). Besides, morphological characteristics of RNR isolates were in line with Nour et al. (1995) who reported similar characteristics for chickpea nodulating bacteria. Also, results obtained in this study were in agreement with that obtained by Jida and Assefa (2012). In addition, Mpepereki et al. (1997) reported colonies which were gummy, translucent and spreading on YMA medium at 2-3 days incubation for root-nodulating bacteria from *Vigna*. It is known that rhizobia are gram-negative, thus Gram staining was used as a confirmative test when isolates are examined under miscroscope. All the isolates in both groups of RNR and NEB were gram-negative. Gauri et al. (2012) also described their chickpea-nodulating isolates as gram-negative. Moreover, Roychowdhury et al. (2015) observed that their strains were white pinkish color and rod-shaped under microscope. They all re-nodulated the host

plants, did not absorb congo red in YMA and did not grow with GPA or ketolactose which are all distinctive features of rhizobial species (Wani et al., 2009; Jida and Assefa, 2012). However, 9 isolates of the RNR group showed unexpected growth with GPA and positive results with ketolactose. When grown on YMA media with bromothymol blue dye, our isolates with no exception turned the color of the indicator to yellow indicating acidic pH and fast growth rates. This was in line with previous reports about the nodulating symbionts of chickpea (Nour et al., 1994; Datta et al., 2015).

On the other hand, the non-nodulating group isolates (NEB) showed morphological and non-nodulability characteristics of the non-rhizobial identity. As the colonies were yellow to yellowish white, or slightly pink mucoidy, with medium to larger diameters, fast growers, and did not induce any nodules on chickpea plants in re-nodulability assays. Similarly, previous works on NEB (Khalifa et al., 2016; Demissie et al., 2018) reported colony characteristics and growth rates for non-nodulating nodule endophytes from soybean, common bean and alfalfa. All the NEB isolates showed gram-negative reactions and appear as rods under microscope (Aserse et al., 2013). They were also fast growers and did not absorb congo red on YMA (Demissie et al., 2018). It is so customary in recent researches that all the isolates (RNR and NEB) residing insides chickpea nodules are being investigated and identified. This is not only exclusive to chickpea but expands to include several leguminous plants (Deng et al., 2011; Aserse et al., 2013; Degefu et al., 2013; Demissie et al., 2018).

5.2 Phenotypic Characterizations

Recently, phenotypic and genotypic invistigations are applied in parallel to obtain highly robust classifications of a particular microbial community. However, the phenotypic characterizations are still essential in terms of selecting candidates that display promising characters in adaptations to harsh stresses like antibiotic, heavy metal, and thermotolerant features (Howieson and Dilworth, 2016).

5.2.1 Utilization of Carbon and Nitrogen Sources

Nutritional requirements of root-nodule bacteria are considered as basic criteria for characterization and identification of rhizobia (Chakrabarti et al., 1981). Root-nodule

bacteria show significant differences concerning utilizations of carbon and nitrogen sources. Our isolates in both nodulating RNB and non-nodulating NEB groups showed variable growth with the tested sources of carbon. The RNB Isolates showed variable growth with starch, sorbitol and citrate while utilized all the other sources of carbon. Chickpea root-nodule bacteria (RNB) were reported to utilize different carbon sources (Nour et al., 1994; L'taief et al., 2007). Also, the variable types of utilized carbohydrates can also be used as a diagnostic feature (Hameed et al., 2004; Küçük and Kivanç, 2008). In line with our results, El-Idrissi et al. (1996) studied rhizobia isolated from root-nodues of the *Caratonia siliqua*. They found that their strains were able to metabolize a wide range of sugars as sole carbon sources. Additionally, Mpepereki et al. (1997) found that indigenous fast strains isolated from nodules of cowpea utilized all the tested 12 sole carbon sources. Fast-growing isolates in this study metabolized numerous sources as sole carbon sources. It is reported that rhizobia strains with fast growth rates possess an oxidative enzyme activity and thought to utilize a broader range of carbon sources (Sadowsky et al., 1983) which coincided with our results. The ability of citrate metabolism as a single carbon source was thought to be confined in the slow-growers Bradyrhizobia (Graham and Parker, 1964). On the other hand, inability of citrate metabolism was observed among many root-nodule bacteria. For instances, results from numerous works reported that isolates from *Vicia* and *Medicago* were unable to utilize citrate (Belay, 2006; Shimekite, 2006; Tsegaye et al., 2015). However, the fast-growing isolates in the current study obviously showed the ability of chickpea nodule bacteria of citrate metabolism as a single source of carbon. Moreover, Datta et al. (2015) reported positive citrate with some nodulating strains of chickpea and other legumes. In contradiction with findings of Küçük and Kıvanç (2008) whose chickpea nodulating bacteria were not able to use starch and citrate, some of the isolates succeeded to utilize both of them. De Oliveria et al. (2007) also observed that the nodulating strains obtained from different legumes could utilize starch. In addition, in the study done by Tsegaye et al. (2015), more than 70% of the strains succeeded to utilize starch to meet their needs of carbon for growth, which is compatible with our findings. Besides, starch was found to serve as a carbon source in many strains of rhizobia. For example, in two different studies, more than 75% of isolates from *Vicia faba* and about 70% from *Lathyrus sativus* (Adal, 2009; Argaw, 2012) were reported to utilize starch. Also, in line with our results, utilization of citrate by chickpea-

nodulating bacteria was reported (Gauri et al., 2012; Wani and Khan, 2013). For the NEB isolates in this study, they all utilized mannitol, glucose, galactose and maltose but utilized starch, citrate and sorbitol at a lesser degree. The findings in this study that NEB isolates were able to metabolize the greater number of caron sources were in contradiction to those obtained by many scientists about NEB strains (Lengyel et al., 2005). However, like our results, Demissie et al. (2018) found 30% of their chickpea non-nodulating strains succeeded to metaboliz the whole carbon sources used in their approach. Some isolates in our study in both groups (NEB and RNR) were able to utilize fructose as a single carbon source which is in line with the findings of others (Keneni et al., 2010). Also, while sorbitol was not utilized by any isolate of the nodulating RNR group, some isolates such as Ad2NEB, Kah1NEB, Afy5NEB and Mers10NEB in the NEB group were able to utilize sorbitol which coincided with previous findings (Khalifa et al., 2016; Demissie et al., 2018).

Among nutritional requirements of rhizobia is the utilization of nitrogen sources. The nitrogen requirements of rhizobia can be met via inorganic salts (e.g. nitrate) or through organic sources like amino acids and peptides of short chains (Jordan, 1984). Our isolates in both groups of RNB and NEB utilized different amino acids (yeast ext, glycine, alanine and tryptophan) and other nitrogenous substrates (Urea and $NaNO_3$) as sole N-sources which correlates with previous studies on chickpea nodule bacteria (Amarger et al., 1997). Moreover, it was observed that all the assessed nodulating strains from common bean were able to utilize numerous amino acids like L-tyrosine, and methionine (Argaw, 2012; Tsegaye et al., 2015). Such a broad line of options in nitrogen source utilizations by rhizobia was previously reported (El-Akhal et al., 2009). In soils deficient in nitrogen, the capability of strains from a particular microbial community to use more than one amino acid as a single source of nitrogen can give an advantage for better endurance and growth (Jida and Assefa, 2012).

Certain amino acids as glycine (Jordan, 1984), asparagine and L-methionine (Zhang et al., 1991) may be inhibitory for root-nodule bacterial growth. However, our isolates in both groups of RNB and NEB utilized glycine, asparagine and L-methionine as single sources of N_2. This is correlated to the study of Demissie et al. (2018) where the isolates in the great number (96%) were able to utilize L-Asparagine. Also, Mohamed et al. (2000) found that the later three amino acids can be utilized as sole nitrogen sources by some root-nodule isolates from *Acacia* spp.

Moreover, our results are in line with that of Küçük and Kıvanç, (2008) who also found that all the tested amino acids including glycine, asparagine and L-methionine were assimilated by the chickpea RNB.

5.2.2 Salinity, Temperature and pH Tolerance

Salinity does not only affect the growth of plants but also inhibits the proliferation and activity of native microorganisms like root-nodule bacteria and also affect the introduced bacteria when applied to soils as inoculants (Singleton et al., 1982). Regarding salinity stress in this study, all isolates in both groups of RNB and NEB tolerated 1 and 2% salt which is compatible with findings on chickpea nodule bacteria (L'ataief et al., 2007; Jida and Assefa, 2012). The fact that root-nodulating bacteria were not affected by low and moderate levels of salinity was reported previously for chickpea nodulating bacteria (Rabie and Alamadini, 2005). This fact was endorsed in many other legumes; Helemish and El-Gammal (1987) examined the impact of NaCl on the survival of *leguminosarum* TAL 271 and reported that this strain was toleratant to low NaCl levels (1 and 2%). Also, Yang et al. (2008) isolated 54 strains of rhizobia from mungbean from different geographical regions of China and found that most of these strains showed salt tolerance at low NaCl concentrations (1%). Additionally, the growth of different cowpea rhizobia isolates at different concentrations of NaCl was studied by Rai et al. (2013) and showed that most strains tolerated 1-2% NaCl. Ability of rhizobia isolates of bean to tolerate increased concentration of NaCl was investigated (Küçük et al., 2006). The study found that all isolates were capable to tolerate 1% NaCl. Moreover, in our stud, 10 isolates from the nodulating RNB group viz. Ada10, Adi4, Adi12A, Adi16A, Amas7, Cor7, Tok1 and Tok4, Esk7B and Afy5 grew at NaCl 5%. Highly saline-tolerant root-nodule *Mesorhizobium ciceri* was also observed (Soussi et al., 2001; Singh et al., 2015). Tolerance of our isolates to high salinity confirmed the conclusion that fast-growing rhizobia are salt tolerant (El Sheikh and Wood, 1989). Küçük et al. (2006) demonstrated rhizobia strains that grew variably at 5% NaCl. Despite originating from the same site, isolates Adi12A and Adi14 had different maximum tolerance at 1 and 5% salt, respectively. Similarly, Maâtallah et al. (2002) observed variations in salt tolerance with chickpea *Mesorhizobia* from the same site. For the non-nodulating NEB isolates, they showed higher tolerance than the RNB isolates as 100% of the isolates tolerated NaCl up to 4% and 11 isolates tolerated up to 5 %. Similarly,

tolerance of high NaCl concentrations was reported for non-nodulating root endophytes; the NEB isolate MSR1 from alfalfa studied by Khalifa et al. (2016), survived at low concentrations of salt while growth decreased after 2% NaCl. At concentrations above 3% NaCl, the strain grew vey poorly and failed to show any growth beyond 4% NaCl. In addition, this strain was able to withstand salinity to eight percent when subjected to increasing dozes of salinity. Additionally, in line wth our NEB isolates, the tolerance of NEB strains to high levels of salinity was observed (Dastager et al., 2014). Utilization of salt tolerant root-nodule bacteria strains contribute to the reclamation of salt-affected soils. Therefore, the ability of root-nodule bacteria to tolerate salinity in on synthetic lab nutritive growth media is an indicative that these bacteria could be candidates for effective symbiosis with host plants in alkaline-saline soils (Singleton et al., 1982).

In connection with temperature stress, the temperature impact on growth of root-nodule bacteria is unhidden (Alexandre et al., 2009; Niste et al., 2013). The optimal range of temperature that support the growth of many strains of rhizobia was considered between twenty-eight and thirty-one degrees Celsius (Graham 1992). Most of our isolates in the RNB group grew at 20-35°C. Maatallah et al. (2002) described a similar maximum temperature growth at 20–35°C for chickpea nodulating bacteria. Besides, all the 28 nodulating isolates of chickpea nodules studied by Küçük et al. (2008) from Turkish soils, succeeded to grow in yeast extract mannitol agar media up to 37°C and the most of these isolates tolerated high temperatures; growing at 40°C. The maximum growth temperature for chickpea rhizobia (Both for *M. ciceri* and *M. mediterraneum*) was reported to be 40°C (Nour et al., 1994, 1995). Like observations of Soussi et al. (2001) on chickpea mesorhizobia, some of our RNR isolates (Adi1, Afy1, Esk7B, Gsehinb and Kir19) showed thermotolerant features as they had optimum temeratures between 35-45°C. The success of certain isolates of rhizobia to show thermotolerant features on synthetic lab media and have high optimal temperatures is useful and is a predication of a possible application of these isolates in soils where high temperatures represent a challenge for survival. This is because the survival of rhizobia isolates at high temperatures on culture media is so relevant to their symbiosis with legume hosts in the field experiments (Hungria et al., 2000). In line with our thermotolerant isolates, the majority of *Vigna unguiculata* rhizobia strains, isolated from tropical arid soils of

in Africa, survived at forty degrees Celsius (Eaglesham and Ayanaba, 1984). Similarly, most of the isolates from common bean endured at high temperatures up to 45°C (Karanja and Wood, 1988). Some isolates (Ada5A, Ada9, Ada10, Adi5, Adi6, Adi16A1, Adi17 and Afy3) in the same group RNR grew at 15°C in tune with Rai et al. (2012) who observed the survival of chickpea mesorhizobia at 15°C and 42°C. Also, cryotolerant rhizobia were found to survive in a viable stautus at low temperatures (Caudry-Reznick et al., 1986). For non-nodulating NEB group, the isolates with no exceptions grew at a range of 15-35°C and 6 isolates succeeded to tolerate up to 50°C NaCl. This correlated to the findings of Demissie et al. (2018) in which about forty percent of the examined strains was thermotolerant; endured at high temperatures up to forty degrees Celsius. Also, the optimum temperature for their isolates was between twenty and thirty degrees Celsius, while 6 strains were cryotolerant (survived at 5°C).

In accordance to pH tolerance, it was observed by Deora and Singhal (2010) that a slight variation in the medium pH might enormously affect the growth of rhizobia. Brockwell et al. (1982) reported that the pH value may be a main restrictive agent in survial of many bacteria in soil. 80% of our isolates, in the nodulating RNB group, grew at pH 5 and they all grew at pH between 6 and 8. Similar findings (Nour et al., 1994; L'taief et al., 2007) reported that chickpea mesorhizobia could exhibit moderately acidic and alkaline pH tolerance (grew well between pH 5 and 8). Also, Mohamed et al. (2000) found that most nodulating isolates from *Acacia* spp. grew at acidic and alkaline pH values. In contrast to Baoling et al. (2007) where no growth was observed at pH 9, 84 and 53% of our isolates tolerated pH 9 and 10, respectively, in coincidence with previous findings (Nour et al., 1994) where chickpea *M. ciceri* strains were generlayy found to have a higher range of tolerance to pH than rhizobia; as they can survive within 4-10 pH. Moreover, Singh et al. (2015) reported alkalotolerant chickpea mesorhizobia that grew well at pH 10. The ability of chickpea rhizobia to withstand in media with alkaline and acidic pH values may be attributed to their ability to bring the pH of the medium to a neutral point (Içgen et al., 2002). For the non-nodulating NEB isolates, they showed alkalotolerant nature as all the isolates succeeded to grow between 6-10 pH while 6 out of 13 isolates were able to grow at pH 5. Our findings correlated to the observations from previous works (Jida and Assefa, 2012) on chickpea root-nodule bacteria. They

found 100% of chickpea strains survived in a vaiable status at slightely acidic and alkanine pH values. Also, Demissie et al. (2018), in line with our findings, found that 5% of his chickpea root-nodule bacteria grew well at alkaline media up to pH 10, whereas 72% was capable of survivng at a slightly higher pH. Taking into consideration that acidity and alkalinity of soil could be critical to the existence and the ability of a microbial population to grow. The examined isolates that show tolerance for pH changes over a wider scope are certainly preferable over those with a limited pH scope when selecting isolates as inoculants. However, the existence and survivability of any elected candidates as inoculant is dependent on their application practices in field.

5.2.3 Antibiotic Resistance and Heavy Metal Tolerance

Sensitivity to different antibiotics, at different ranges of concentrations, varied between species and it was suggested that such variation may be a useful taxonomic tool (Somasegaran and Hoben, 1994). In the antibiotic resistance tolerance tests, our isolates in both of nodulating RNB and non-nodulating NEB groups, significantly resisted most of the tested antibiotic concentrations in variable degrees. Most of the isolates in RNB group showed insensitivity to all the assessed antibiotics. The highest tolerance was in the order, NA > Str > KA > Tetr > Amp > Ery. Considering that all the isolates in this study were fast growers, this contradicted what was reported by Jordan et al. (1994) that fast-growing root-nodule bacteria were sensitive to a broad scope of antibiotics and could resist only a narrow scope of antibiotics. Also, in contradiction to our results, Mpepereki et al. (1997) studied the antibiotic sensitivity of cowpea nodule isolates from 14 Zimbabwean soils. They found that the intrinsic antibiotic sensitivity was generally higher in fast-growers compared to slow-growers. However, many observations from different works supported our findings. For example, Gauri et al. (2012) studied the resistance of 40 isolates from chickpea root nodules against many antibiotics and 25% of the isolates showed high insensitivity to the assessed antibiotics. Also, in line with our results, most strains of *Phaseolus* were insensitive against many antibiotics like kanamycin (10) and streptomycin (40) Küçük et al. (2006). In another study for the same author, Küçük et al. (2008) found that the chickpea nodule isolates displayed a good degree of insensitivity to numerous antibiotics which correlated to our results. Moreover, the evaluation by Gida and Assefa (2012) of AR of *Cicer* root-nodule to antibiotics

clarified that many of the evaluted strains displayed good insenitivity to NA, erythromycin, ampicillin, Chloroamphenicol, neomycin and streptomycin. Most of the rhizobial cultures tested by Tsegaye et al. (2015) obviously survived against most of the evaluated concentrations of antibiotics and that the resistence of most strains declined with the increment of the dozes of antibiotics supplied in the growth medium.

Interestingly, in our study, nalidixic acid (50), tetracycline (20) and streptomycin (50) µg ml^{-1} were 100% tolerated by all isolates of our RNB isolates. With respect to non-nodulating isolates, they showed higher resistance to the assessed antibiotics than recorded for the RNB isolates. They all 100% were resistant to the minimum concentrations in µg ml^{-1} of NA (50), Tetra (20), and Stre. (50). Moreover, more than 60% of the isolates were able to show sensitivity to the other higher concentrations of the evaluated antibiotics. This is consistent with findings on non-nodulating endophytes from chickpea and other nodule bacteria. For example, In the study by Khalifa et al. (2016), the strains from alfalfa displayed AR gainst 100% of the dozes of the evaluated antibiotics but for chloramphenicol. This is in coincidence with findings on non-nodulating bacteria from the Glycine max (Ramesh et al., 2014) who investigated the. Moreover, the study by Demissie et al. (2018) on the endophytic bacteria in chickpea nodules, displayed differences responses to the evaluated antibiotics. The insensitivity of nodule-bacteria against is considered as an advantage for better adaption and durability of these isolates when introduced to soils as inoculants for biofertilization.

Antibiotic resistance assays might be exploited for identifying microbial strains within legume nodules when investigating the degree survivalbility in a competitive environmental condition (Kremer and Peterson, 1982). Moreover, variations within strains of a bacterial population could be identified through AR of the strains (Somasegaran and Hoben, 1994). Consequently, AR patterns might serve as subsidiary tool for distinguishing variuos isolates (Amarger et al., 1997; Küçük et al., 2008). It has been reported that the number and/or activities of soil microorganisms were reduced in soils contaminated with heavy metals (Hao et al., 2014). Several studies had shown that heavy metals might have been toxic to root-nodule bacteria when present in soil in moderate to high concentrations. Lakzian et al. (2002) examined *R. leguminosarum* bv. *viciae*, collected from soil contaminated with

varying concentrations of heavy metals. They found that the lowest rhizobial populations were present in the soil with the highest concentration of heavy metals.

For HM resistance, our isolates were tolerant to Hg (100%), Cr (88%) and Cu (81%) at their low concentraions (10 µg ml^{-1}). High tolerance to Cr and Cu was also observed in previous findings for chickpea root-nodule bacteria and rhizobia from other legumes (Jida and Assefa, 2012; Wani et al., 2013). Also, like our results, previous studies reported the tolerance of rhizobial strains against mercury (Carrasco et al., 2005). Adaptations and surviavabilty of root-nodule bacteria to HM in synthetic media may be attributed to their isolation from soil contaminated with heavy meatals. The high level of resistance to certain HM might serve to elect promising isolates for field applications (Sinclair and Eaglesham, 1984). With respect to the non-nodulating isolates, they showed more tolerance to the same concentrations of the heavy metals than did the isolates in the nodulating group. Our isolates in the NEB group showed 100% resistance to low concentrations of 10 µg ml^{-1} of Zn, Cu, Hg, Ni and Cr. Moreover, more than 90 of the NEB isolates were resistant to the higher concentrations of these metals. Similarly, Demissie et al. (2018) reported that 50 and 26% of root nodule isolates were able to withstand copper and zinc, respectively. The correlation between tolerance of bacterial strains to HM on synthetic media and their capability to withstand in soils where they can be introduced as inoculants was well invistiagted (Alikhani and Yakhchali, 2010). Also, different responses of root-nodule bacteria to heavy metals can be considered as basic criteria for differentiation and identification of these bacteria (Biomy, 2000).

5.3 Biochemical Activities

Biochemical activities of root-nodule bacteria were a normal tool in their characterization in many previous and recent works (Bhagat et al., 2014; Bhatt and Vyas, 2014). Regarding the biochemical activities, our isolates in both the nodulating and non-nodulating group showed positive results for catalase, oxidase, urease, 3-ketolactase while none of them was positive to amylase or methyl red. However, some isolates in the non-nodulating group showed gelatinase activity. Also, some isolates in both groups showed positive indole acetic acid production. Many studies reported positive activities for catalase and oxidase tests in chickpea nodulating bacteria and other legumes (Naz et al., 2009; Datta et al., 2015). Also, Sadowsky et

al. (1983) evaluated the biochemical activities of several symbioant bacteria of soybean and observed that they showed catalase, oxidase, and urease positivity, in line with our results. The positive catalase activity is and advantage for the producing bacteria as the former enables bacteria to alleviate and mitigate the impact of free radicles formed because of oxidative stresses. Since free radicals have harmful effect on the plant growth, possessing of positive activit for this enzyme might help increase plant growth through catalase activity could promote plant growth indirectly (Bumunang and Babalola, 2014). However, the study of Sadowsky showed that some isolates were positive to gelatinase which opposed our results here. Additionally, Shahzad et al. (2012) revealed, in contrast to some of our isolates in both groups, that none of their 50 nodule bacteria from alfalfa was positive to urea or indole acetic acid. Like our observations, Hunter (2007) reported negative gelatinase activity is a feature of root-nodulating bacteria. Besides, Niste et al. (2015) reported negative gelatinase activity for all the studied isolates from alfalfa and soybean which correlated to our results. Also, the same study revealed positive observations for urease activity in many of the tested isolates in line with some of our isolates which showed positive urease activity. For the non-nodulating isolates, 100% of the isolates were catalase, indolee and oxidase positive. Like our results, Demissie et al. (2018) observed that thirty percent of the tested isolates excreted catalase. Besides, in the work by Trivedi et al. (2011) more than half of the tested isolates succeeded to produce IAA. The ability to produce IAA by nodulating bacteria was described in many invitigatigations and due to this ability serve to increase the lenghth of lateral roots and root hairs and hence help promote plant growth (Okon and Kapulnik, 1986; Sridevi and Mallaiah, 2007; Etesami et al., 2009). Also, IAA production is well reported in the endophytic non-nodulating bacteria. For example, endophytic isolates from alfalfa were reported to produce IAA (Khalifa et al., 2016). In contrast to our results, it was observed in other works that root-nodule bacteria (nodulating or endophytes) were positive for amylase (Bhatt and Vyas, 2014; Demissie et al., 2018).

5.4 Clustering of Isolates on Phenotypic Basis

The final matrix contained 120 isolates in the nodulating RNR group (Fig. 3.17) and 13 isolates in the non-nodulating endohytes NEB (Fig. 3.18). 71 traits, including the nutritional, physiological and biochemical parameters were assessed for both groups and a phenogram was drawn separately for each group. Observations were recorded

one and zero for positive and negative, repectively, and hierarchical clustering analysis was carried out for each group isolates using IBM SPSS version 23 software. The pearson correlation interval measure and between-groups linkage were used for the hierarchical clustering. Results revealed diversity of chickpea root nodule rhizobial and non-rhizobial endophytic isolates in Turkey. Such heterogeneity was reported before (Demissie et al., 2018). Cluster analysis placed RNR isolates into 3 clusters at 25% similarity. 113 strains came within cluster I. The cluster I isolates belonged to different origins and utilized most of the sole sourcs of carbon and nitrogen. They moderately tolerated 1 to 3% NaCl and pH between 5 and 10 and were sensitive to temperatures above 35°C. They showed high tolerance to Cr, Cd, Zn, Ni, NA and KA and were IAA and urease positive. Cluster I isolates displayed similar phenotypic features to *Mesorhizobium ciceri*, based on the previous observations on chickpea root-nodule (Jarvis et al., 1982; Nour et al., 1994, 1995). Cluster 2 had 5 isolates namely; Adi1, Afy1, Esk7B, Gsehinb and Kir19. They displayed higher tolerance to salinity and temperature than isolates in cluster I as they all grew at 3% Na and grew optimally between 35 and 45°C. They also tolerated all concentrations of Zn, tetracycline and streptomycin and were all urease positive. According to the results of cluster 2 isolates and previous describtions of Jarvis et al. (1982) and Nour et al. (1994, 1995) they displayed similar features with *M. mediterraneum*. Two isolates namely Adi15 and Afyon5 fell into cluster 3. Unlike other isolates, cluster 3 isolates did not utilize starch as a sole source of C, but grew well with sorbitol and citrate and endured salinity up to 4%. They all grew optimally between 20-40°C and withstand all concentrations of Ni, Cd, NA and Str., but were sensitive to Amp. Based on these traits and description of Jarvis et al. (1982), Nour et al. (1994, 1995) they showed a close relationship with *Mesorhizobium* sp. clustering of isolates did not correlate with their sites of collections. Atypical example for this was isolates Afy1 and Kir19 which came from diverse sites but clustered together in cluster 2. For the non-nodulating isolates, they came also with 3 clusters. Cluster 1 came with 4 isolates viz. Ada2NEB, Kah1NEB, Kah3NEB and Kir13NEB. Cluster 2 had 4 isolates, to wit, Ada5NEB, Afy1NEB, Afy5NEB and Kut9NEB. Cluster 3 comprised 5 isolates namely, Cor5NEB Cor7NEB Kir11NEB Kir12NEB and Mers10NEB.

5.5 Molecular Characterizations

5.5.1 REP-PCR and 16S rDNA Sequencing and Phylogeny

The identification and taxonomy of root-nodule bacteria has been tradionally based on the phenotypic characterizations (Eardly et al., 2005). However, it has been argued (Demezas et al., 1991) that the reclassification and identification of the genus Rhizobium should be carried out based on molecular characterizations rather than the phenotypic characteristics or plant bacterial specificity. It was stated before that it was one of the aims of this research was to precisely determine the identity of the non-nodulating endopphytes in the Turkish chickpea root nodules.

In conformity with our aims and to launch a more precise taxonomy of the of the isolates in the non-nodulating group (NEB) in this study, further molecular characterization using REP-PCR and 16S rDNA-based phylogeny were used along with the phenotype-based characterizations of this group. We performed REP-PCR patterns for the nodulating isolates just to compare the diversity obtained in the PCR patterns with that obtained from the phenotypic characterizations and thus we did not identify those isolates from the nodulating group using the 16S rRNA but we did identify representatives from the NEB isolates only. The REP-PCR patterns obtained for the nodulating RNR isolates showed high resolution patterns. The clusters of these patterns did not put the isolates in the same clusters as obtained in the phenotypic characterizations. This is normal because the phenotypic and molecular characterizations are not necessarily the same. For the 13 non-rhizobial isolates, the REP-PCR displayed very characteristic bands (Fig. 3.24) and random representatives were selected for sequencing the 16S rDNA. When used to amplify the REP sequences distributed along the DNA, REP1R-1 and REP2-1 primers anneal optimaly at the desired sequences to help amplify the sequences and result in obvious patterns which can be used to categorize and fingerprint the isolates. This confirmed what was reported by De Bruijn (1992) that REP-units amplifications with the primers REP-IR-1 and REP-2 is a simple endorsed tool for fingerprinting root-nodule bacteria due to the dvantages of REP-PCR: (a) There is no need to specific sequenced probes to detect the REP-units, and one pair of primers is enough to amplify sequences of similar or divergent species. (b) The REP patterns are easy to resolve through ordinary electrophoresis and no need to extra techniques to analyze the REP-PCR results (Gilson et al., 1984). They are DNA sequences have been

observed within the genome of both gram-negative like rhizobia, and gram-positive bacteria like bacillus (Stern et al., 1984). The present study confirmed this fact that the products of the REP-PCR, with chromosomal DNA of the different isolates as template, were found to generate very characterisic patterns when separated on agarose gels. These DNA repetitive segments contain sequences whose mutations are very rare and highly stable throughout the life span of bacteria. The exact task of these sequences remains a controversial point. There are many functions are claimed to be correlated to these units; they serve to make mRNA more stable, have a role during crossing-over in chromosomes, in transitional coupling between genes, homologous recombination, and other reported functions (Higgins et al., 1982; Newbury et al., 1987; Stern et al., 1988; Gilson et al., 1990; Shyamala et al., 1990). No particular task is obviously attached to REP-units or can explain the stability of these DNA segments or their distribution along the genome (Hulton et al., 1991). There are theories which attribute the existence and propagation of these REP-elements to the conversion of genes (Higgins et al., 1988). The DNA sequence of these conserved inverted repeats in REP elements has facilitated the conclusion of REP sequences in all organisms (Hulton et al., 1991). These consensus sequences were used by Versalovic et al. (1991) to synthesize REP-specific oligonucleotide primers and also to probe the DNA of a variety of microorganisms for the presence of REP-like sequences using polymerase chain reaction (Mullis et al., 1987). There are many studies used REP units as a tool of genotyping many bacterial species. For example, in the approach by Tajima et al. (2000), REP-PCR was the choice to identify and calssify different bacterial genera from different legumes in Japan, through highly resolved patterns of REP-units which proved to be a robuost tool for be reproducible. The isolates included the bacteria genera *Bradyrhizobium*, *Sinorhizobium*, and *Rhizobium* strains. Also, (REP-PCR) was used to cluster 70 root-nodule bacteria strains, collected from root nodules of the wild legume *Hedysarum* from Morocco, into 30 REP-PCR groups (Ezzakkioui et al., 2015). Besides, Soybean nodule isolates were characterized using REP-PCR to study the intra-species diversity of rhizobia in 3 regions of China (Zhang et al., 2016). In addition, the REP-PCR was used as the fingerprinting option for 20 isolates from Algeria to determine the taxonomic position of and the evaluation of the level of approximation or divergence between these strains and the reference strains belonging to different genera of rhizobia (Benselama et al., 2018). It is worth mentioning that REP-PCR

has been frequently used for fingerprinting of non-nodulating nodule endophytes. For example, genetic diversity among 15 isolates of *B. thuringiensis*, isolated from nodules of 6 leguminous plants was determined by REP-PCR (Mishra et al., 2017). These studies revealed the existence of these REP-elements within the genome of various nodule bacteria and thereby support and extend the data presented by Versalovic et al. (1991) on the apparently ubiquitous nature of these elements in bacteria. Similarly, our study confirmed the applicability of the REP-PCR method for characterization of related root-nodule bacterial strains. This agreed with results obtained by De Bruijin et al. (1992) concerning the applicability of REP-PCR method for the identification of related root-nodule strains. Our results also support the conclusion of Versalovic et al. (1991) that REP-PCR could become a powerful means for the molecular genetic analysis of diversity within a microbial population, because it renders the basis for clustering strains within genera and species and could help to determine the phylogenetic relationships, as demonstrated here with a preliminary analysis of our isolates. Advances in molecular methods have allowed reviewing the taxonomic classification of many bacterial groups (including RNB), mostly inferred from the 16S rDNA sequence (Young et al., 2004). In the last ten years, sequencing of 16S rRNA genes was considered the most advanced method for investigating the taxonomy and analyzing the phylogeny of bacterial populations including root-nodule bacteria. This has resulted in the addition of new genera and species to the root-nodule community. Now, performing the 16S rRNA gene sequencing has become an ordinary procedure for the identification of any bacterial species (including root-nodule bacteria) (Rivas et al., 2004). This molecular technique of identifying bacteria has been used alongside with phenotypic and other characterizations in what was called the polyphasic taxonomy or it could be used alone as a robust alternative method for the non-molecular characterization methods of bacteria (Demissie et al., 2000). Unlike the other non-molecuar methods which might give variable data due to differences in expressions of the assessed traits (like phenotypic characterizations), the 16S rRNA gene sequencing provides a clear robust identification and reproducible data even for scarce isolates (Weisburg et al., 1991). Another reason explaines the advantages of the 16S rRNA is that the universal databases of the identified sequences are being shared world-wide (Woese et al., 1987; Van de Peer et al., 2000).

In our study, we used the 16S rDNA sequences of 8 representative isolates (Ad5NEB, Afy5NEB, Cor5NEB, Kah7NEB, Kir12NEB, Kir20NEB, Kut9NEB and Mers10NEB) selected from the REP-PCR fingerprinting of the 13 non-rhizobia endophytes from the cultivated Turkish chickpea nodules to support the taxonomic classification. Dependent on sequence similarity of the 16S rRNA genes of the representative strains to sequences in the database using NCBI BLAST software, the isolate Ad5NEB was identified with 99% sequence coincidence with *Rahnella aquatilis*. *Rahnella* was reported before as a nodule endophyte in many works in coincidence with our results. For instance, fifty-five isolates from different legumes collected from thirty-one geographical sites in Ethiopia, were identified based on 16S rRNA by Aserse et al. (2013). Rahnella, along with other 11 endophytic genera, was reported to be one of the endophytic genera. Also, De Meyer et al. (2015) studied 654 NEB isolates of indigenous leguminous plants in Belgian soils. The results of 16S rRNA sequencing indicated that these NEB isolates were highly diverse and *Rahnella* was dominant inside the root nodules of *Robinia pseudoacacia*. The study also reported the co-ccurrence of *Rahnella* with the genus Mesorhizobium. Also, Bahroun et al. (2018). Investigated 16 endophytic bacterial isolates from broad bean and chickpea nodules. The studied revealed that 6.25% of the isolates were affiliated to *Rahnella aquatilis*. Additionally, Saidi et al. (2011) observed nodule endophytic isolates that were affiliated to *Rahnella* in his study of 104 nodule bacteria from faba bean from Tunisia. The study interestingly revealed that isolates assigned to *Rahnella* showed a nifH -like amplification product. The results from 16S rRNA gene sequencing of 5 endophytic representative isolates in this study viz. Afy5NEB, Kir12NEB, Kir20NEB, Kut9NEB and Mers10NEB, revealed that they all belong to the genus Enterobacter. Isolates Afy5NEB, Kir12NEB, Kir20NEB, Kut9NEB recorded 99% similarity to Enterobacter cloacae and Mers10NEB showed 99% similarity to *Enterobacter* spp.. Thus, *Enterobacter* was the dominant endophyte in our study. There are many works strengthen the literature on the endophytic nature of *Enterobacter* inside the nodules of chickpea and other legume plants. For example, *Enterobacter cloacae* was observed as an endophyte within nodules from numerous leguminous plants such as *Arachis hypogaea*, *Conzattia multiform* and numerous wild legumes in Algeria (Benhizia et al., 2004; Wang et al., 2006; Taurian et al., 2010). Also, Jakson et al. (2017) reported that nodule endophytic bacteria from *Vigna unguiculata* were highly diverse where *Enterobacter* spp. was abundant.

Moreover, Aserse et al. (2013), through 16S rRNA gene sequencing, identified most of his 55 nodule endophytic isolates as Gram-negative bacteria where 19 isolates were identified as *Enterobacter*. Additionally, the existence of *Enterobacter* within *Arachis hypogaea* nodules was also observed by Ibáñez et al. (2009). Moreover, Medicago sativa root-nodules were revealed to have numerous endophytic bacteria including *Endobacter* as observed by Ramırez-Bahena et al. (2013).

Recently, Khalifa et al. (2016) identified the MSR1 strain from alfalfa root-nodules as *Enterobacter cloacae* based on both biochemical and 16S rRNA characterizations. In the study of 6 sex endophytes from soybean root-nodules, they were found to belong to five different genera including *Enterobacter* (Zhao et al., 2018). Also, Tariq et al. (2014) phylogenetically identified his strain MSP10 from pisum sativum as *Enterobacter* dending on 16S rRNA gene analysis. Furthermore, Bahroun et al. (2018) found that 12.5% of the 16 endophytic nodule bacteria from broad bean and chickpea were affiliated to *Enterobacter*. In our study, the Cor5NEB isolate was identified as *Pseudomonas koreensis* with similarity 99%. Among Gram-negative soil bacteria, *Pseudomonas* is one of the soil-borne geam-negative bacteria and is considered as the genus with the highest density in the rhizosphere (Bardas et al., 2009). Like the results in our study, De Meyer et al. (2015) studied 654 isolates obtained from 30 indigenous legume plants in Belgium. They revealed that *Pseudomonas* represented (15.9%) of the genera inside the root nodules. In addition, the investigation of the strain Zong1 from Sophora alopecuroides by Zhao et al. (2013) revealed that it was closely related to the genus *Pseudomonas*. Besides, Zhao et al. (2018) isolated numerous nodule endophytes from Glycinemax from China and the molecular identification revealed they belonged to many genera including *Psudomonas*. In addition, Beghalem et al. (2017) investigated the endophytic bacterial diversity within nodules of 2 leguminous plants of the genus *Sulla*. Phenotypic and molecular analyses revealed that the endophytes were related to *Pseudomonas*. Also, in the study by Bahroun et al. (2018), nodule endophytes from studied a broad bean and chickpea. The study revealed that 68.75% of the isolates were affiliated to *Pseudomonas*. Finally, in a study by Sharma et al. (2012) *Pseudomonas* spp. was the dominant species among 22 endophytes within nodules from chickpea and moth bean. The isolate Kah7NEB was identified with 99% similarity to *Rhizobium nepotum*. In line with our results, the fact that some rhizobia

could exist inside nodules as endophytes without nitrogen fixing or enter with the main true nodulating rhizobia was reported in some researches.

Rhizobia have been known to possess the ability to induce nodule formation. However, they have been reported in numerous research articles to exist inside nodules without trigerring any nodule formation (Lupwayi et al., 2004; Deng et al., 2011). In line to our results, 8 isolates of 55 endophytic nodule bacteria from different woody, shrub and food legumes were identified as Rhizobium (Aserse et al., 2013). Also, *Rhizobium tropici* and *Rhizobium* were described as non-nodulating endophytes within bean nodules in Ethiopian soils (Aserse et al., 2012). Also, non-nodulating strains of rhizobia were found in soybean nodules (Wu et al., 2011). When found, non-nodulating rhizobia were found to lack the nod genes (e.g. nodC, nodA, or nodD) (Aserse et al., 2013). In addition, fifty-five isolates from thirty-one geographical areas in Ethiopia were identified by Aserse et al. (2013). *Mesorhizobium*, along with other 11 endophytic genera, was reported to be one of the endophytic genera that give a negative nodulation assay. Also, 81 endophytic isolates from different *Acacia* nodules in Algeria and were identified to belong to 9 genera including *Pseudomonas, Rhizobium* and *Agrobacterium* (Boukhatem et al., 2016).

CHAPTER VI
CONCLUSIONS

From the results obtained in this study, it can be concluded that mesorhizobia are the main chickpea-nodulating bacteria as indicated by the morphological, physiological and biochemical results of 120 isolates collected from different agricultural soils in 17 cities in Turkey. Namely, *Mesorhizobium ciceri*, *Mesorhizobium mediterraneum,* and *Mesorhizobium* sp. were represented by 113 isolates in cluster 1, 4 isolates in cluster 2 and 4 isolates in cluster 3, respectively. The results in this study in their major part coincided with the previously reported data on chickpea-nodule bacteria with some variations that might have indicated the nature of the environmental conditions in the soils where the isolates were collected. Similarly, the results obtained from phenotypic and 16S rRNA gene sequences for chickpea non-symbiotic endophytes in this study revealed that they were included in 4 genera namely *Rahnella aquattilis, Enterobacter cloacae, Pseudomonas koreensis* and *Rhizobium nepotum*. These genera were also found to exist as nodule endophytic non-symbionts in many legume plants including chickpea.

In the present study, isolates that showed promising criteria like utilization of numerous substrates as sole sources of carbon and nitrogen and tolerance to stress conditions such as pH, salinity, temperature…etc. could be good candidates for future usage as biofertilizer inocula in the field practices to increase the yield of chickpea at low costs.

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APPENDIX

A.1 Results of Phenotypic Characterizations

In the following tables, see list of abbreviations and table 3.1, for short forms of the phenotypic traits and the codes of sites of collections, respectively**.**

Table A.1.1 Growth (1) and no growth (0) of RNR isolates with carbon and nitrogen sources. Traits that were the same for the whole isolates were omitted

Isolate	Starch	Citrate	Sorbitol	Trypt.	Urea	KNO3
Usak1	0	0	0	1	1	1
Usak2	1	1	0	1	1	0
Usak13	0	1	0	1	1	0
Usak17	1	1	0	1	1	0
Yoz2	0	0	0	1	0	0
Yoz6	0	0	0	1	0	0
Yoz7	0	0	0	1	0	0
Yoz8	0	0	0	1	1	0
Yoz11	0	0	0	1	1	0
Yoz14	0	0	0	1	1	0
Yoz15	1	1	1	1	0	0
Yoz16	1	1	1	1	1	0
Kir12	1	1	0	1	1	1
Kir15	0	0	0	0	0	0
Kir17A	0	1	0	1	1	1
Kir19	1	1	0	0	0	0
Kir20	0	0	0	1	1	1
Kir21	1	1	0	0	0	0
Kon2	0	1	0	0	0	0
Kon3	1	1	1	1	1	1
Kon7	0	0	0	1	1	0
Kon9	1	1	1	1	1	1
Kut2	0	0	0	1	0	1
Kut9	0	0	0	0	0	0
Mer1	0	0	0	0	0	0
Mer2	0	0	0	1	0	0
Mer3	0	0	0	1	0	0
Mer4	0	0	0	1	0	1
Mer5A	0	0	0	1	0	1
Mer5B	0	0	0	0	0	0
Mer6A	0	1	1	1	1	1
Mer6B	0	1	1	1	1	1

Table A.1.1 Continued

Isolate	Starch	Citrate	Sorbitol	Trypt.	Urea	KNO3
Mer7	0	0	0	1	1	1
Mer8	0	1	1	1	0	0
Mer9	0	0	0	1	0	1
Mer10A	0	0	0	1	0	1
Mer11	1	1	0	1	0	1
Mer12	1	1	0	1	0	1
Mer13	0	0	0	1	0	0
Mer16	1	1	1	1	1	0
Mer17	0	0	0	1	1	0
Mer18	0	1	0	1	1	1
Mer26	1	1	0	1	1	1
Tok1	0	1	0	1	1	1
Tok2	0	0	0	1	1	1
Tok3	0	0	0	1	1	0
Tok4	0	0	0	1	1	1
Urf2	0	0	0	1	1	0
Cor9	0	0	0	1	0	1
Esk6	0	0	0	0	0	0
Esk7A	0	0	0	0	0	0
Esk7B	0	0	0	1	1	1
GBak	0	0	0	0	0	0
GBur1A	1	1	1	0	0	0
GBur1B	0	1	1	1	1	0
GBur3	1	1	1	1	1	1
Gogu1	0	0	0	1	0	1
Gogu2	0	0	0	0	0	0
Gsar1A	0	0	0	0	0	0
Gsehinb	1	1	1	0	0	0
Gsehitk1	0	1	0	0	0	0
Gsenlik1	1	1	1	1	1	1
Gsut2	1	1	0	1	1	1
Gyav1	0	0	0	0	0	0
Gyav3A	0	0	0	1	1	0
Gyes1	0	0	0	1	0	0
Hat1	0	0	0	1	1	1
Hat2	0	0	0	0	0	0
Hat3	0	0	0	1	1	1
Hat4A	0	0	0	1	1	0
Kah1	0	1	1	1	0	0
Kah2A	1	1	1	1	1	1
Kah3	0	0	0	1	1	1
Kah6	1	1	1	0	0	0
Kah7	0	1	0	0	0	0
Kah8	1	1	1	1	1	1
Kah9	0	0	0	1	0	0

Table A.1.1 Continued

Isolate	Starch	Citrate	Sorbitol	Trypt.	Urea	KNO3
Kah10	0	0	0	1	1	1
Kah13	0	0	0	1	1	1
Kir1	1	1	1	0	0	0
Kir4	0	1	0	0	0	0
Kir5A	1	1	0	0	0	0
Kir9	0	0	0	1	0	0
Kir10	1	1	0	1	1	0
Ada2	1	0	0	0	0	0
Ada5A	1	0	0	1	1	1
Ada6A	0	0	0	1	0	0
Ada8	0	1	0	1	1	0
Ada9	0	1	0	0	0	0
Ada10	0	0	0	0	0	0
Adi1	0	0	1	1	0	1
Adi4	0	0	0	1	0	0
Adi5	0	0	0	1	1	0
Adi6	0	1	0	1	1	1
Adi7	0	0	0	0	0	0
Adi9	1	0	0	1	1	1
Adi12A	0	1	1	1	0	0
Adi14	1	0	0	1	1	0
Adi15	0	1	1	1	1	1
Adi16A1	0	0	0	0	0	0
Adi17	1	1	1	1	1	1
Afy1	0	0	0	0	0	0
Afy3	1	0	0	1	1	0
Afy5	0	1	1	0	0	0
Aks1	0	0	0	0	0	0
Aks2	0	0	0	0	0	0
Aks3A	1	1	1	0	0	0
Aks3B	0	0	0	1	1	0
Aks4A	0	0	0	1	1	0
Aks5	1	1	1	1	0	0
Aks7	0	0	0	1	1	0
Aks10	1	1	0	1	0	1
Amas 3	0	1	0	1	1	1
Amas 4	1	1	0	1	1	1
Amas 7	0	0	0	0	0	0
Cor2A	1	1	1	1	1	0
Cor3	0	0	0	1	0	0
Cor4	0	0	0	1	1	0
Cor5	0	0	0	0	0	0
Cor7	0	0	0	1	1	1

Table A.1.2 Growth (1) and no growth (0) of RNR isolates at different salt (Na) concentrations and temperature degrees (Tm). Traits that were the same for the whole isolates were omitted

Isolate	Na 3	Na 4	Na 5	Tm 15	Tm 20	Tm 25	Tm 30	Tm 35	Tm 40	Tm 45	Tm 50
Ada2	0	0	0	0	0	0	1	1	0	0	0
Ada5A	1	0	0	1	1	1	1	1	0	0	0
Ada6A	1	0	0	0	1	1	1	1	0	0	0
Ada8	0	0	0	0	0	0	1	0	0	0	0
Ada9	1	0	0	1	1	1	1	1	0	0	0
Ada10	1	1	1	1	1	1	1	1	1	1	0
Adi1	1	1	0	0	1	1	1	1	1	1	0
Adi4	0	0	0	0	0	1	1	0	0	0	0
Adi5	1	1	1	1	1	1	1	0	0	0	0
Adi6	1	1	0	1	1	1	1	1	1	0	0
Adi7	1	1	0	0	0	0	1	1	1	0	0
Adi9	0	0	0	1	1	1	1	1	1	0	0
Adi12A	1	1	1	0	0	1	1	1	1	0	0
Adi14	0	0	0	0	1	1	1	1	1	0	0
Adi15	1	1	0	1	1	1	1	1	1	0	0
Adi16A1	1	1	1	1	1	1	1	0	0	0	0
Adi17	0	0	0	1	1	1	1	1	1	1	0
Afy1	1	0	0	0	0	0	1	1	1	1	0
Afy3	1	0	0	1	1	1	1	1	0	0	0
Afy5	1	1	1	0	1	1	1	1	1	0	0
Aks1	0	0	0	0	1	1	1	1	1	0	0
Aks2	0	0	0	1	1	1	1	1	1	1	0
Aks3A	0	0	0	0	1	1	1	1	1	1	0
Aks3B	0	0	0	1	1	1	1	1	0	0	0
Aks4A	1	0	0	1	1	1	1	1	0	0	0
Aks5	1	0	0	0	1	1	1	1	1	0	0
Aks7	1	0	0	0	0	0	1	1	0	0	0
Aks10	1	0	0	1	1	1	1	0	0	0	0
Amas 3	0	0	0	1	1	1	1	1	0	0	0
Amas 4	0	0	0	0	1	1	1	1	0	0	0
Amas 7	1	1	1	0	0	1	1	1	1	0	0
Cor2A	1	0	0	0	1	0	1	1	0	0	0
Cor3	1	1	0	0	0	0	1	0	0	0	0
Cor4	0	0	0	1	1	1	1	1	1	1	0
Cor5	0	0	0	1	1	1	1	1	1	0	0
Cor5	1	1	1	1	1	1	1	1	0	0	0
Cor7	1	0	0	1	1	1	1	0	0	0	0
Cor9	1	0	0	1	1	1	1	0	0	0	0
Esk6	1	0	0	0	0	1	1	0	0	0	0
Esk7A	1	1	1	1	1	1	1	1	1	1	0
Esk7B	1	0	0	0	1	1	1	1	0	0	0
GBak	1	1	1	1	1	1	1	0	0	0	0

Tm= temperature

Table A.1.2 Continued

Isolate	Na 3	Na 4	Na 5	Tm 15	Tm 20	Tm 25	Tm 30	Tm 35	Tm 40	Tm 45	Tm 50
GBur1A	1	0	0	0	1	1	1	1	1	0	0
GBur1B	1	1	0	0	1	1	1	1	0	0	0
GBur3	0	0	0	1	1	1	1	0	0	0	0
Gogu1	0	0	0	0	0	0	1	1	0	0	0
Gogu2	1	0	0	0	1	1	1	1	0	0	0
Gsar1A	1	1	0	0	1	1	1	1	1	1	0
Gsehinb	1	1	0	0	0	0	1	1	1	1	0
Gsehitk1	1	1	0	1	1	1	1	1	0	0	0
Gsenlik1	1	0	0	1	1	1	1	0	0	0	0
Gsut2	1	1	0	0	0	1	1	1	1	1	0
Gyav1	1	0	0	0	1	1	1	1	0	0	0
Gyav3A	1	1	0	0	1	1	1	1	0	0	0
Gyes1	0	0	0	1	1	1	1	1	0	0	0
Hat1	0	0	0	1	1	1	1	0	0	0	0
Hat2	0	0	0	1	1	1	1	1	1	0	0
Hat3	1	1	0	1	1	1	1	1	0	0	0
Hat4A	0	0	0	1	1	1	1	0	0	0	0
Kah1	0	0	0	0	0	0	1	1	0	0	0
Kah2A	1	1	0	0	0	1	1	1	0	0	0
Kah3	1	1	0	0	1	1	1	1	0	0	0
Kah6	0	0	0	1	1	1	1	1	1	0	0
Kah7	1	1	0	0	1	1	1	1	1	0	0
Kah8	1	1	0	1	1	1	1	1	0	0	0
Kah9	1	1	0	0	1	1	1	1	0	0	0
Kah10	0	0	0	0	1	1	1	1	1	0	0
Kah13	0	0	0	0	1	1	1	1	0	0	0
Kir1	1	1	0	0	1	1	1	1	0	0	0
Kir4	1	1	0	1	1	1	1	0	0	0	0
Kir5A	0	0	0	1	1	1	1	0	0	0	0
Kir9	1	1	0	1	1	1	1	1	0	0	0
Kir10	1	1	0	0	1	1	1	1	0	0	0
Kir12	0	0	0	0	1	1	1	1	0	0	0
Kir15	1	1	0	1	1	1	1	1	1	0	0
Kir17A	1	1	0	0	0	0	1	1	0	0	0
Kir19	1	0	0	0	0	0	1	1	1	1	0
Kir20	1	0	0	0	0	1	1	0	1	0	0
Kir21	1	1	0	0	0	1	1	1	0	0	0
Kon2	1	1	0	0	1	1	1	1	0	0	0
Kon3	1	0	0	0	0	1	1	0	0	0	0
Kon7	1	0	0	0	1	1	1	1	1	1	0
Kon9	1	0	0	0	1	1	1	1	1	0	0
Kut2	1	1	0	0	1	1	1	1	1	0	0
Kut9	1	1	0	0	0	1	1	1	0	0	0

Table A.1.2 Continued

Isolate	Na 3	Na 4	Na 5	Tm 15	Tm 20	Tm 25	Tm 30	Tm 35	Tm 40	Tm 45	Tm 50
Mer1	1	0	0	0	0	1	1	1	0	0	0
Mer2	1	0	0	0	0	0	1	1	0	0	0
Mer3	1	0	0	0	0	1	1	0	0	0	0
Mer4	0	0	0	0	0	1	1	1	0	0	0
Mer5A	1	1	0	0	0	0	1	1	0	0	0
Mer5B	1	1	0	0	0	1	1	1	0	0	0
Mer6A	0	0	0	0	1	1	1	1	0	0	0
Mer6B	1	0	0	0	1	1	1	0	0	0	0
Mer7	1	1	0	0	1	1	1	1	0	0	0
Mer8	0	0	0	0	0	1	1	1	0	0	0
Mer9	1	1	0	0	1	1	1	1	0	0	0
Mer10A	0	0	0	0	1	1	1	1	1	0	0
Mer11	0	0	0	0	0	1	1	1	0	0	0
Mer12	0	0	0	0	1	1	1	1	0	0	0
Mer13	0	0	0	0	1	1	1	1	0	0	0
Mer16	0	0	0	0	1	1	1	1	1	0	0
Mer17	1	1	0	0	1	1	1	1	0	0	0
Mer18	1	1	0	0	0	1	1	1	0	0	0
Mer26	1	1	0	0	1	1	1	1	0	0	0
Tok1	1	1	1	0	1	1	1	1	0	0	0
Tok2	0	0	0	0	0	1	1	1	0	0	0
Tok3	1	1	0	0	1	1	1	1	0	0	0
Tok4	1	1	1	0	1	1	1	0	0	0	0
Urf2	1	1	0	0	1	1	1	1	0	0	0
Usak1	0	0	0	0	1	1	1	1	0	0	0
Usak2	1	1	0	0	1	1	1	1	0	0	0
Usak13	0	0	0	0	1	1	1	0	0	0	0
Usak17	1	1	0	0	0	1	1	0	0	0	0
Yoz2	1	1	0	0	1	1	1	1	0	0	0
Yoz6	0	0	0	0	1	1	1	1	0	0	0
Yoz7	0	0	0	0	0	1	1	1	0	0	0
Yoz8	0	0	0	0	1	1	1	1	0	0	0
Yoz11	0	0	0	0	1	1	1	1	0	0	0
Yoz14	0	0	0	0	0	1	1	1	0	0	0
Yoz15	0	0	0	0	1	1	1	1	0	0	0
Yoz16	0	0	0	0	0	1	1	1	0	0	0

Table A.1.3 Growth (1) and no growth (0) of RNR isolates at different pH values and heavy metal concentrations (µg ml^{-1}) and temperature degrees (Tm). Traits that were the same for the whole isolates were omitted

Isolate	pH 5	pH 9	pH 10	Zn 10	Zn 20	Zn 50	Cu 10	Cu 50	Cu 100	Hg 20	Hg 50
Usak1	0	1	1	1	1	1	1	0	0	1	0
Usak2	1	1	0	1	1	1	1	1	1	0	0
Usak13	1	1	1	0	0	0	0	0	0	0	0
Usak17	1	1	1	1	1	1	1	1	1	0	0
Yoz2	1	1	1	0	1	1	1	0	0	1	0
Yoz6	1	1	0	1	1	1	1	0	0	1	0
Yoz7	1	0	0	1	1	1	1	1	1	0	0
Yoz8	1	1	0	1	1	0	1	1	1	0	0
Yoz11	1	1	0	1	1	0	1	0	0	0	0
Yoz14	0	1	0	1	1	0	1	0	0	0	0
Yoz15	1	1	0	1	1	0	1	0	0	0	0
Yoz16	1	1	0	1	1	0	1	0	0	0	0
Kir12	0	0	0	0	0	0	1	1	1	0	0
Kir15	1	0	0	0	0	0	1	1	1	0	0
Kir17A	1	1	0	1	1	0	1	1	1	0	0
Kir19	1	0	0	1	1	1	0	0	0	1	0
Kir20	0	1	0	1	1	1	0	0	0	1	0
Kir21	1	0	0	1	0	0	1	1	1	1	0
Kon2	0	1	1	1	0	0	1	0	0	1	0
Kon3	1	1	1	1	1	1	1	1	1	1	0
Kon7	1	1	1	1	1	1	1	1	1	1	0
Kon9	1	1	1	1	1	0	1	1	0	1	0
Kut2	0	1	1	1	1	1	1	1	1	0	0
Kut9	1	1	1	1	0	0	1	0	0	0	0
Mer1	1	1	1	0	0	0	1	0	0	0	0
Mer2	0	1	1	0	0	0	1	1	1	0	0
Mer3	1	1	1	1	1	1	1	1	1	1	1
Mer4	1	1	1	1	1	0	0	0	0	0	0
Mer5A	1	1	1	1	1	1	0	0	0	1	1
Mer5B	1	1	0	1	1	1	0	0	0	0	0
Mer6A	1	1	0	0	0	0	1	1	1	0	0
Mer6B	1	1	0	1	1	1	1	1	1	0	0
Mer7	1	1	0	0	0	0	1	1	1	1	1
Mer8	1	0	0	1	1	1	0	0	0	0	0
Mer9	1	1	1	1	1	0	1	1	1	1	1
Mer10A	0	1	0	1	1	1	1	1	1	0	0
Mer11	1	0	0	1	1	0	0	0	0	0	0
Mer12	1	1	0	1	1	0	1	1	1	1	1
Mer13	1	1	1	1	1	0	1	1	0	0	0
Mer16	1	1	0	1	0	1	1	0	0	1	1
Mer17	1	1	0	1	1	0	1	0	0	1	1
Mer18	1	1	0	1	0	1	1	1	1	1	1

Table A.1.3 Continued

Isolate	pH 5	pH 9	pH 10	Zn 10	Zn 20	Zn 50	Cu 10	Cu 50	Cu 100	Hg 20	Hg 50
Mer26	1	1	1	1	1	1	1	1	1	1	1
Tok1	1	1	1	1	0	0	1	0	0	1	1
Tok2	1	1	1	1	0	0	1	1	1	1	1
Tok3	1	1	0	1	0	0	1	1	1	1	1
Tok4	1	1	0	1	1	1	1	1	1	1	1
Urf2	1	1	0	0	0	0	1	0	0	1	1
Cor9	1	1	0	1	1	1	1	1	1	1	1
Esk6	1	1	0	1	1	1	0	0	0	1	1
Esk7A	1	1	1	0	0	0	1	1	1	0	0
Esk7B	0	0	0	1	1	1	0	0	0	0	0
GBak	1	1	0	1	1	1	1	0	0	0	0
GBur1A	1	1	1	0	0	0	1	1	1	1	1
GBur1B	1	0	0	1	1	1	1	1	1	1	1
GBur3	1	1	1	1	1	1	1	1	1	1	1
Gogu1	1	1	1	1	0	0	1	1	1	1	1
Gogu2	1	0	0	1	1	1	1	1	1	1	1
Gsar1A	0	1	0	1	0	0	1	0	0	1	1
Gsehinb	1	1	1	1	1	1	1	0	0	1	1
Gsehitk1	1	1	1	1	1	1	1	0	0	1	0
Gsenlik1	1	1	0	1	1	0	1	0	0	0	0
Gsut2	1	1	0	1	1	1	1	1	1	0	0
Gyav1	0	1	1	1	0	0	1	1	0	0	0
Gyav3A	1	1	1	1	1	1	1	1	1	0	0
Gyes1	0	1	1	1	0	0	1	1	1	0	0
Hat1	1	1	1	1	1	1	1	1	0	0	0
Hat2	1	1	1	1	0	0	1	1	1	0	0
Hat3	1	1	0	1	0	0	1	1	1	0	0
Hat4A	1	1	0	1	1	1	1	1	0	1	1
Kah1	1	1	0	1	0	0	1	1	1	0	0
Kah2A	1	1	1	1	1	1	1	1	1	0	0
Kah3	0	1	1	0	0	0	1	1	0	1	1
Kah6	1	1	1	1	1	1	1	1	1	0	0
Kah7	0	1	1	0	0	0	1	1	1	0	0
Kah8	1	1	1	1	1	1	1	1	0	1	1
Kah9	0	1	1	1	1	1	1	1	0	0	0
Kah10	1	1	1	1	0	0	1	1	0	0	0
Kah10	1	1	1	1	0	0	1	1	0	0	0
Kah13	1	1	0	1	0	0	1	1	1	0	0
Kir1	1	1	0	1	1	1	0	0	0	0	0
Kir4	1	0	0	1	1	1	0	0	0	1	1
Kir5A	1	0	0	0	0	0	1	1	1	1	1
Kir9	1	0	0	1	1	1	1	1	1	1	1
Kir10	1	0	0	1	1	1	1	1	1	0	0
Kir12	0	0	0	0	0	0	1	1	1	0	0

Table A.1.3 Continued

Isolate	pH 5	pH 9	pH 10	Zn 10	Zn 20	Zn 50	Cu 10	Cu 50	Cu 100	Hg 20	Hg 50
Kir15	1	0	0	0	0	0	1	1	1	0	0
Kir17A	1	1	0	1	1	0	1	1	1	0	0
Kir19	1	0	0	1	1	1	0	0	0	1	0
Kir20	0	1	0	1	1	1	0	0	0	1	0
Kir21	1	0	0	1	0	0	1	1	1	1	0
Kon2	0	1	1	1	0	0	1	0	0	1	0
Kon3	1	1	1	1	1	1	1	1	1	1	0
Kon7	1	1	1	1	1	1	1	1	1	1	0
Kon9	1	1	1	1	1	0	1	1	0	1	0
Kut2	0	1	1	1	1	1	1	1	1	0	0
Kut9	1	1	1	1	0	0	1	0	0	0	0
Mer1	1	1	1	0	0	0	1	0	0	0	0
Mer2	0	1	1	0	0	0	1	1	1	0	0
Mer3	1	1	1	1	1	1	1	1	1	1	1
Mer4	1	1	1	1	1	0	0	0	0	0	0
Mer5A	1	1	1	1	1	1	0	0	0	1	1
Mer5B	1	1	0	1	1	1	0	0	0	0	0
Mer6A	1	1	0	0	0	0	1	1	1	0	0
Mer6B	1	1	0	1	1	1	1	1	1	0	0
Mer7	1	1	0	0	0	0	1	1	1	1	1
Mer8	1	0	0	1	1	1	0	0	0	0	0
Mer9	1	1	1	1	1	0	1	1	1	1	1
Mer10A	0	1	0	1	1	1	1	1	1	0	0
Mer11	1	0	0	1	1	0	0	0	0	0	0
Mer12	1	1	0	1	1	0	1	1	1	1	1
Mer13	1	1	1	1	1	0	1	1	0	0	0
Mer16	1	1	0	1	0	1	1	0	0	1	1
Mer17	1	1	0	1	1	0	1	0	0	1	1
Mer18	1	1	0	1	0	1	1	1	1	1	1
Mer26	1	1	1	1	1	1	1	1	1	1	1
Tok1	1	1	1	1	0	0	1	0	0	1	1
Tok2	1	1	1	1	0	0	1	1	1	1	1
Tok3	1	1	0	1	0	0	1	1	1	1	1
Tok4	1	1	0	1	1	1	1	1	1	1	1
Urf2	1	1	0	0	0	0	1	0	0	1	1

Table A.1.4 Growth (1) and no growth (0) of RNR isolates at different heavy metal and antibiotic concentrations (µg ml^{-1}). Traits that were the same for the whole isolates were omitted

Isolate	Ni 10	Ni 50	Ni 100	Cd 10	Cd 20	Cr 10	Cr 25	Cr 50	NA 50	NA 100
Ada2	0	0	0	0	0	1	1	1	1	1
Ada5A	1	0	0	0	0	0	0	0	1	0
Ada6A	1	1	0	1	1	1	1	1	1	1
Ada8	1	1	0	1	1	1	1	1	1	1
Ada9	1	0	0	1	1	1	1	1	1	1
Ada10	1	0	0	1	1	1	1	1	1	1
Adi1	1	0	0	0	0	1	1	1	1	0
Adi4	1	1	0	1	1	1	1	1	1	1
Adi5	1	0	0	1	0	0	0	0	1	1
Adi6	1	1	1	1	1	1	1	0	1	1
Adi7	0	0	0	1	1	1	1	1	1	1
Adi9	1	1	0	0	0	1	1	1	1	0
Adi12A	1	1	0	1	1	1	1	0	1	1
Adi14	0	0	0	1	0	1	1	1	1	1
Adi15	1	1	1	1	1	1	0	0	1	1
Adi16A	1	1	1	0	0	1	1	1	1	1
Adi17	1	0	0	1	1	1	0	0	1	1
Afy1	1	0	0	0	0	1	1	0	1	0
Afy3	1	1	1	1	1	0	0	0	1	1
Afy5	1	1	1	1	1	1	1	1	1	1
Aks1	1	0	0	0	0	1	1	1	1	1
Aks2	1	0	0	1	1	1	1	1	1	0
Aks 3A	1	0	0	1	1	1	0	0	1	1
Aks3B	1	1	1	1	0	1	1	1	1	1
Aks 4A	1	1	1	1	0	1	1	0	1	1
Aks5	0	0	0	1	1	1	1	1	1	1
Aks7	1	1	1	1	1	1	1	1	1	1
Aks10	1	1	1	1	1	1	1	1	1	0
Amas 3	1	1	1	1	1	1	1	0	1	1
Amas 4	1	1	1	1	0	1	1	1	1	1
Amas 7	0	0	0	1	1	1	0	0	1	0
Cor2A	1	1	0	1	1	1	1	1	1	1
Cor3	1	1	0	0	0	1	1	1	1	1
Cor4	1	1	0	1	1	1	1	0	1	0
Cor5	1	1	1	1	1	1	1	1	1	1
Cor7	0	0	0	0	0	1	1	0	1	1
Cor9	1	1	1	1	1	1	1	1	1	1
Esk6	1	0	1	1	0	1	1	0	1	1
Esk7A	1	0	1	1	1	1	1	1	1	1
Esk7B	1	1	0	0	0	1	1	1	1	0
GBak	1	1	0	1	1	1	1	1	1	1
GBur1A	1	1	1	1	1	1	1	1	1	1
GBur1B	0	0	0	1	1	1	1	0	1	1

Table A.1.4 Continued

Isolate	Ni 10	Ni 50	Ni 100	Cd 10	Cd 20	Cr 10	Cr 25	Cr 50	NA 50	NA 100
GBur3	1	1	1	0	0	1	1	1	1	1
Gogu1	1	1	1	1	1	1	1	1	1	1
Gogu2	1	1	0	1	1	1	1	1	1	1
Gsar1A	1	1	0	0	0	1	1	0	1	1
Gsehinb	1	1	1	0	0	1	1	1	1	1
Gsehit1	0	0	0	1	1	1	1	0	1	1
Gsenlik1	0	0	0	1	1	1	0	0	1	1
Gsut2	1	1	1	1	0	1	1	0	1	1
Gyav1	1	0	0	1	0	1	1	1	1	1
Gyav3A	1	0	0	1	0	1	1	1	1	1
Gyes1	1	0	0	1	0	1	1	1	1	1
Hat1	1	1	1	1	1	1	1	0	1	1
Hat2	0	0	0	1	0	1	1	0	1	1
Hat3	1	1	1	1	1	1	0	0	1	1
Hat4A	1	1	0	1	1	1	1	1	1	1
Kah1	1	1	1	1	1	1	1	0	1	1
Kah2A	1	1	1	1	0	1	1	1	1	1
Kah3	0	0	0	1	0	1	1	1	1	1
Kah6	0	0	0	1	1	1	0	0	1	1
Kah7	1	0	1	1	0	1	1	1	1	1
Kah8	1	1	1	0	1	1	1	1	1	1
Kah9	0	0	0	1	1	1	1	1	1	1
Kah10	1	1	1	1	0	1	1	1	1	1
Kah13	1	1	1	1	1	1	1	1	1	1
Kir1	0	0	0	1	0	1	0	0	1	1
Kir4	0	0	0	1	1	1	1	1	1	1
Kir5A	1	1	1	1	0	1	0	0	1	1
Kah9	0	0	0	1	1	1	1	1	1	1
Kah10	1	1	1	1	0	1	1	1	1	1
Kah13	1	1	1	1	1	1	1	1	1	1
Kir1	0	0	0	1	0	1	0	0	1	1
Kir4	0	0	0	1	1	1	1	1	1	1
Kir5A	1	1	1	1	0	1	0	0	1	1
Kir9	1	1	1	0	1	1	1	1	1	1
Kir10	1	0	0	1	0	1	1	1	1	1
Kir12	1	0	0	1	1	1	1	0	1	1
Kir15	1	0	0	1	1	1	1	1	1	1
Kir17A	1	1	1	1	1	1	0	0	1	1
Kir19	1	1	1	1	0	1	0	0	1	1
Kir20	0	0	0	0	0	1	1	1	1	1
Kir21	1	1	1	1	0	1	1	0	1	1
Kon2	1	0	0	1	1	1	0	0	1	1
Kon3	1	0	0	1	1	1	1	1	1	1
Kon7	1	1	1	1	0	1	0	0	1	1

Table A.1.4 Continued

Isolate	Ni 10	Ni 50	Ni 100	Cd 10	Cd 20	Cr 10	Cr 25	Cr 50	NA 50	NA 100
Kon9	0	0	0	1	0	1	1	1	1	1
Kut2	0	0	0	1	0	1	1	1	1	1
Kut9	1	1	1	1	0	1	0	0	1	1
Mer1	0	0	0	1	1	1	1	1	1	1
Mer2	1	1	1	1	1	1	0	0	1	1
Mer3	1	1	1	1	0	1	1	1	1	1
Mer4	1	1	0	1	0	1	1	1	1	1
Mer5A	1	0	0	1	1	1	1	0	1	1
Mer5B	1	0	0	1	1	1	1	1	1	1
Mer6A	1	1	0	1	0	1	0	0	1	1
Mer6B	1	1	0	1	1	1	1	0	1	1
Mer7	1	1	0	1	1	1	1	0	1	1
Mer8	1	1	0	1	1	1	0	0	1	1
Mer9	1	0	1	1	1	1	1	1	1	1
Mer10A	1	0	1	1	1	1	1	1	1	1
Mer11	1	0	1	1	1	1	1	1	1	1
Mer12	1	0	1	1	0	1	1	1	1	1
Mer13	1	0	0	1	1	1	0	0	1	1
Mer16	1	0	0	1	0	1	1	1	1	1
Mer17	1	0	1	1	0	1	1	1	1	1
Mer18	1	1	1	1	0	1	0	0	1	1
Mer26	1	1	1	1	1	1	1	1	1	1
Tok1	1	0	0	1	1	1	1	1	1	1
Tok2	1	1	0	1	0	1	1	1	1	1
Tok3	1	1	0	1	1	1	0	0	1	0
Tok4	1	1	1	1	0	1	1	1	1	1
Urf2	1	1	1	1	1	0	0	0	1	1
Usak1	1	1	1	0	0	1	1	1	1	1
Usak2	1	1	1	1	1	1	0	0	1	1
Usak13	1	0	0	1	1	1	0	0	1	1
Usak17	1	0	0	1	1	1	1	1	1	0
Yoz2	1	1	1	1	1	1	1	1	1	1
Yoz6	1	1	1	1	1	1	1	1	1	1
Yoz7	1	1	1	1	1	1	1	1	1	1
Yoz8	1	0	0	1	1	1	1	1	1	1
Yoz11	1	0	0	1	1	1	1	1	1	1
Yoz14	1	0	0	0	0	1	1	0	1	0
Yoz15	1	0	0	0	0	1	1	0	1	0
Yoz16	1	0	0	0	0	1	1	0	1	0

Table A.1.5 Growth (1) and no growth (0) of RNR isolates at different heavy antibiotic concentrations (µg ml^{-1}) and positive (0) or negative (0) activity of some biochemical tests. Traits that were the same for the whole isolates were omitted

Isolate	Tetr 50	Amp 50	Amp 100	Ery 25	Str 100	KA 50	KA 100
Usak1	1	1	1	0	0	1	1
Usak2	1	1	0	1	1	1	1
Usak13	1	1	1	1	1	1	1
Usak17	1	1	1	0	0	1	1
Yoz2	0	1	0	1	1	1	1
Yoz6	0	1	1	1	1	1	1
Yoz7	0	1	1	1	0	1	1
Yoz8	0	1	0	1	1	1	1
Yoz11	0	1	1	0	1	0	0
Yoz14	0	1	0	0	1	1	1
Yoz15	1	1	0	1	1	0	0
Yoz16	1	1	0	0	1	0	0
Kir12	0	1	1	1	1	0	0
Kir15	1	0	0	1	1	1	1
Kir17A	1	1	1	1	1	1	1
Kir19	1	1	1	0	1	1	0
Kir20	1	1	0	1	1	1	1
Kir21	1	1	1	1	1	1	1
Kon2	1	0	0	0	1	1	0
Kon3	0	1	1	1	0	1	1
Kon7	1	1	1	1	1	1	1
Kon9	1	1	1	0	1	0	1
Kut2	1	1	1	1	1	0	1
Kut9	0	1	1	1	1	0	1
Mer1	1	0	0	1	1	1	1
Mer2	1	1	1	0	0	1	1
Mer3	1	1	1	1	1	1	1
Mer4	1	1	1	1	1	1	1
Mer5A	1	0	0	0	1	1	0
Mer5B	1	1	0	1	1	1	1
Mer6A	0	1	0	1	1	1	1
Mer6B	0	0	0	0	0	1	0
Mer7	1	1	1	1	1	1	0
Mer8	1	1	1	1	1	0	0
Mer9	1	1	1	1	0	1	1
Mer10A	0	0	0	1	0	1	1
Mer11	0	1	1	0	1	1	1
Mer12	0	1	1	1	1	1	1
Mer13	0	0	0	1	1	1	1
Mer16	0	1	1	1	0	1	1
Mer17	1	1	1	0	1	1	0

Table A.1.5 Continued

Isolate	Tetr 50	Amp 50	Amp 100	Ery 25	Str 100	KA 50	KA 100
Mer18	1	0	0	0	1	1	1
Mer26	1	1	1	1	1	1	1
Tok1	1	1	0	1	1	1	1
Tok2	0	1	1	1	0	1	1
Tok3	1	1	1	0	1	0	0
Tok4	1	1	0	1	1	0	0
Urf2	1	1	1	1	1	1	1
Cor9	1	1	0	1	1	1	1
Esk6	1	1	1	1	0	0	0
Esk7A	1	0	0	1	1	1	1
Esk7B	1	0	0	0	1	1	1
GBak	1	1	1	0	0	1	1
GBur1A	1	1	1	1	1	1	1
GBur1B	1	1	1	1	1	1	1
GBur3	1	0	0	0	1	0	0
Gogu1	1	1	1	0	0	1	1
Gogu2	0	1	1	1	1	1	1
Gsar1A	1	0	0	1	1	0	0
Gsehinb	1	1	1	1	1	1	1
Gsehitk1	0	1	1	1	0	1	1
Gsenlik1	1	0	0	1	1	1	0
Gsut2	0	1	1	1	1	1	1
Gyav1	1	1	1	1	1	1	1
Gyav3A	1	0	0	1	0	1	1
Gyes1	1	0	0	1	1	1	1
Hat1	0	1	1	1	1	1	1
Hat2	1	0	0	1	1	1	1
Hat3	1	1	1	1	0	1	1
Hat4A	1	1	1	1	0	0	0
Kah1	0	1	1	1	0	0	0
Kah2A	1	0	0	1	1	0	0
Kah3	1	1	1	1	1	1	1
Kah6	0	1	1	1	1	1	1
Kah7	1	0	0	1	0	1	0
Kah8	0	1	1	1	1	1	1
Kah9	1	1	1	1	1	1	1
Kah10	1	0	0	1	1	1	1
Kah13	1	1	1	1	1	1	1
Kir1	1	1	1	1	0	1	1
Kir4	1	1	1	1	1	0	0
Kir5A	1	0	0	0	0	1	1
Kir9	0	1	0	0	1	1	0
Kir10	0	1	0	1	1	1	1
Ada2	1	0	0	1	1	1	0

Table A.1.5 Continued

Isolate	Tetr 50	Amp 50	Amp 100	Ery 25	Str 100	KA 50	KA 100
Ada5A	1	1	1	0	1	0	0
Ada6A	0	1	1	0	0	1	1
Ada8	1	1	0	1	1	1	1
Ada9	0	1	0	1	0	1	1
Ada10	1	1	1	1	1	1	1
Adi1	1	0	0	0	1	0	0
Adi4	1	1	1	1	1	0	0
Adi5	0	1	1	1	0	1	1
Adi6	0	1	1	0	0	1	1
Adi7	1	0	0	1	1	1	1
Adi9	1	1	1	1	1	1	0
Adi12A	1	1	0	1	0	1	1
Adi14	0	1	1	1	1	1	1
Adi15	1	0	0	1	1	0	0
Adi16A1	1	0	0	0	1	1	1
Adi17	0	1	1	0	0	1	1
Afy1	1	1	1	1	1	1	1
Afy3	1	0	0	1	0	1	1
Afy5	0	0	0	1	1	1	0
Aks1	1	1	1	1	1	1	0
Aks2	1	1	1	1	1	0	0
Aks3A	1	1	1	1	1	1	0
Aks3B	1	0	0	1	0	1	1
Aks4A	0	0	0	1	1	1	1
Aks5	1	1	1	1	1	1	1
Aks7	1	1	1	1	1	1	1
Aks10	0	0	0	1	1	1	1
Amas 3	1	0	0	1	1	1	1
Amas 4	1	1	1	1	1	0	0
Amas 7	0	1	1	0	1	1	1
Cor2A	0	0	0	1	0	1	1
Cor3	0	1	1	1	1	1	1
Cor4	0	0	0	1	1	1	0
Cor5	1	1	1	0	1	1	1
Cor7	1	1	0	0	1	1	1

Table A.1.6 Biochemical activities of RNR isolates. Results coded (1) for growth and (0) for no growth. Traits that were the same for the whole isolates were omitted

Isolate	Urease	IAA	lactase	GPA	Isolate	Urease	IAA	lactase	GPA
Usak1	1	0	0	0	Tok1	1	0	0	0
Usak2	0	0	0	0	Tok2	1	0	0	0
Usak13	0	0	0	0	Tok3	0	0	0	0
Usak17	0	0	0	0	Tok4	0	0	0	0
Yoz2	1	0	0	0	Urf2	0	0	0	0
Yoz6	0	1	0	0	Cor9	1	1	0	0
Yoz7	0	0	0	0	Esk6	0	0	0	0
Yoz8	1	0	0	0	Esk7A	0	0	0	0
Yoz11	1	0	0	0	Esk7B	1	0	0	1
Yoz14	1	1	0	0	GBak	0	0	0	0
Yoz15	1	1	0	0	GBur1A	1	0	0	0
Yoz16	1	1	0	0	GBur1B	1	0	0	0
Kir12	1	1	0	0	GBur3	1	1	0	0
Kir15	0	0	0	0	Gogu1	1	0	0	0
Kir17A	0	0	0	0	Gogu2	1	0	0	0
Kir19	1	1	1	1	Gsar1A	1	0	0	0
Kir20	1	0	0	0	Gsehinb	1	0	0	1
Kir21	1	0	0	0	Gsehitk1	1	0	0	0
Kon2	1	0	0	0	Gsenlik1	1	1	1	0
Kon3	1	0	0	0	Gsut2	1	0	0	1
Kon7	1	0	0	1	Gyav1	1	0	0	0
Kon9	1	1	1	0	Gyav3A	1	0	0	0
Kut2	1	0	0	0	Gyes1	1	1	0	0
Kut9	0	0	0	0	Hat1	1	0	0	0
Mer1	1	0	0	0	Hat2	1	1	0	0
Mer2	0	0	0	0	Hat3	1	0	0	0
Mer3	1	0	0	0	Hat4A	0	1	1	0
Mer4	0	0	0	0	Kah1	0	0	0	0
Mer5A	0	0	0	0	Kah2A	1	0	0	0
Mer5B	0	0	0	0	Kah3	1	0	0	0
Mer6A	0	0	0	0	Kah6	1	0	0	0
Mer6B	0	0	0	0	Kah7	1	0	0	0
Mer7	1	0	0	0	Kah8	0	0	0	0
Mer8	0	0	0	0	Kah9	1	1	0	0
Mer9	0	0	0	0	Kah10	0	0	0	0
Mer10A	1	0	0	0	Kah13	1	0	0	0
Mer11	1	0	0	0	Kir1	1	0	0	0
Mer12	0	0	0	0	Kir4	0	1	1	0
Mer13	0	0	0	0	Kir5A	1	0	0	0
Mer16	0	0	0	0	Kir9	0	0	0	0
Mer17	1	0	0	0	Kir10	0	0	0	0
Mer18	0	0	0	0	Ada2	1	1	0	0
Mer26	1	0	0	0	Ada5A	0	1	0	0

Table A.1.6 Continued

Isolate	Urease	IAA	lactase	GPA	Isolate	Urease	IAA	lactase	GPA
Ada6A	0	0	0	0	Aks1	1	0	0	0
Ada8	1	1	1	0	Aks2	0	0	0	0
Ada9	1	0	0	0	Aks3A	1	0	0	1
Ada10	1	0	0	0	Aks3B	0	0	0	0
Adi1	1	0	0	1	Aks4A	1	0	0	0
Adi4	0	1	0	0	Aks5	0	0	0	0
Adi5	1	0	0	0	Aks7	0	0	0	0
Adi6	0	0	0	0	Aks10	1	1	0	0
Adi7	0	0	0	0	Amas 3	1	0	0	0
Adi9	0	0	0	0	Amas 4	0	0	0	0
Adi12A	1	1	0	0	Amas 7	1	0	0	0
Adi14	1	0	0	0	Cor2A	1	1	1	0
Adi15	0	1	1	0	Cor3	1	0	0	0
Adi16A1	1	0	0	0	Cor4	1	0	0	1
Adi17	0	1	1	0	Cor5	1	0	0	0
Afy1	1	0	0	1	Cor7	1	1	0	0
Afy3	0	0	0	0					
Afy5	0	1	0	0	Aks2				

Table A.1.7 Growth (1) and no growth (0) of NEB isolates with different carbon and nitrogen sources. Traits that were the same for the whole isolates were omitted

Isolate	Starch	Citr	Sorb	YE	Gly	Alan	Trypt	Urea	KNO3
Ad2NEB	0	1	1	1	1	1	0	0	0
Ad5NEB	0	0	0	1	1	1	0	0	0
Afy1NEB	0	0	0	1	1	1	0	0	0
Afy5NEB	1	1	1	1	1	1	0	0	0
Cor5NEB	0	0	0	1	1	1	1	1	0
Cor7NEB	0	0	0	1	1	1	1	1	0
Kah1NEB	1	1	1	1	1	1	1	0	0
Kah3NEB	0	0	0	1	1	1	1	1	0
Kir11NEB	1	1	0	1	1	1	1	0	1
Kir12NEB	0	1	0	1	1	1	1	1	1
Kir13NEB	1	1	0	1	1	1	1	1	1
Kut9NEB	0	0	0	1	1	1	0	0	0
Mers10NEB	1	1	1	1	1	1	1	1	0

Citrate, sorbitol, yeast extract, glycine, alanine and tryptophan were coded as Citr, Sorb, YE, Gly, Alan and Trypt, respectively.

Table A.1.8 Growth (1) and no growth (0) of NEB isolates at different salt (Na), temperatures (Tm), pH and heavy metals (µg ml^{-1}). Traits that were the same for the whole isolates were omitted

Isolate	Na5	Tm 40	Tm 45	Tm 50	pH 5	Zn 50	Cu 50	Cu 100	Hg 20	Hg 50
Ad2NEB	1	1	0	0	1	0	0	0	1	0
Ad5NEB	1	1	1	0	0	0	1	1	0	0
Afy1NEB	1	1	1	1	1	1	0	0	1	0
Afy5NEB	1	1	1	1	1	1	0	0	1	0
Cor5NEB	1	1	1	1	0	0	0	0	1	1
Cor7NEB	0	1	1	0	1	0	1	1	1	1
Kah1NEB	1	1	0	1	1	1	0	0	0	0
Kah3NEB	1	0	0	0	1	0	0	0	0	0
Kir11NEB	1	0	0	0	1	1	1	1	1	1
Kir12NEB	1	0	0	0	1	0	1	1	1	1
Kir13NEB	1	0	0	0	0	0	0	0	1	1
Kut9NEB	1	1	1	1	1	1	0	0	1	1
Mers10NEB	0	0	0	0	1	1	1	0	1	1

Table A.1.9 Growth (1) and no growth (0) of NEB isolates at different concentrations of heavy metals and antibiotics (µg ml^{-1}). Traits that were the same for the whole isolates were omitted

Isolate	Ni 50	Ni 100	Cd 10	Cd 20	Cr 25	Cr 50	NA 100	KA 50	KA 100	Tetr 50
Ad2NEB	1	1	1	1	1	1	1	1	0	0
Ad5NEB	0	0	0	0	1	1	1	1	0	1
Afy1NEB	0	0	1	1	1	1	0	0	0	1
Afy5NEB	0	0	1	1	0	0	1	1	0	1
Cor5NEB	1	0	1	0	1	1	1	1	1	1
Cor7NEB	1	0	1	0	1	0	1	1	1	0
Kah1NEB	0	0	1	1	1	1	1	1	1	1
Kah3NEB	1	1	1	1	1	1	1	1	1	1
Kir11NEB	1	0	1	1	1	1	0	1	1	0
Kir12NEB	1	0	1	1	1	0	1	1	1	1
Kir13NEB	1	1	1	0	1	1	1	0	0	1
Kut9NEB	0	0	1	1	0	0	0	1	1	0
Mers10NEB	0	0	1	1	1	1	1	1	1	0

Table A.1.10 Growth (1) and no growth (0) of NEB isolates at different concentrations of heavy metals and antibiotics (µg ml^{-1}) and positive (1) or negative (0) observations of some biochemical tests. Traits that were the same for the whole isolates were omitted

Isolate	Amp. 50	Amp. 100	Ery. 25	Ery. 50	Str. 100	Urease	Gela-tinase	lactase	GPA
Ad2NEB	0	0	1	0	1	0	0	0	0
Ad5NEB	1	1	1	1	1	1	1	0	1
Afy1NEB	1	1	1	1	1	0	1	0	1
Afy5NEB	1	1	1	0	1	1	0	1	1
Cor5NEB	0	0	1	0	0	0	0	1	1
Cor7NEB	0	0	1	1	1	1	0	1	1
Kah1NEB	1	1	1	1	1	0	0	1	1
Kah3NEB	1	1	1	1	1	0	1	1	1
Kir11NEB	0	0	1	0	1	1	1	0	1
Kir12NEB	0	0	1	1	1	1	0	0	1
Kir13NEB	1	1	1	1	1	0	0	0	0
Kut9NEB	1	1	0	0	1	1	0	0	1
Mers10NEB	0	0	1	1	0	1	0	1	1

CURRICULUM VITAE
AHMED MOHAMED AHMED MAHMOUD

Education

University	Faculty	Department	Degree	Year
Bani-Swaif	Science	Botany and Microbiology	B.Sc.	2002-2003
Cairo	Science	Microbiology	Pre-M.Sc.	2003
Bani-Swaif	Science	Microbiology	M.Sc.	2004-2010
Gaziantep	Science and Arts	Biology	Ph.D.	2013-2019

PUBLICATIONS

Hamdi H. Zahran, Rajaa Chahboune, Silvia Moreno, Eulogio J. Bedmar, Medhat Abdel-Fattah, Manal M. Yasser, Ahmed M. Mahmoud (2013). Identification of rhizobial strains nodulating Egyptian grain legumes, *International Microbiology*, **16**, 157-163.

Ahmed Mohamed Mahmoud, Durdane Mart, Canan Can (2018). Phenotypic characterization of indigenous rhizobia nodulating chickpea in Turkey reveals high diversity, *legume Research*, 430, 1-6. DOI: 10.18805/LR-430