CHARACTERIZATION OF VARIOUS *Rhizobium cicer* STRAINS AND ASSESSMENT OF THEIR SYMBIOTIC EFFECTIVENESS

A Master's Thesis

Presented by

Bülent İÇGEN

to

the Graduate School of Natural and Applied Sciences

of Middle East Technical University

in Partial Fulfillment for the Degree of

MASTER OF SCIENCE

in

BIOTECHNOLOGY

MIDDLE EAST TECHNICAL UNIVERSITY

ANKARA

June, 1994

Approval of the Graduate School of Natural and Applied Sciences.

for

Prof. Dr. İsmail TOSUN

Director

I certify that this thesis satisfies all the requirements as a thesis for the degree of Master of Science.

Prof. Dr. Nesrin HASIRCI

Chairman of the Department

We certify that we read this thesis and that in our opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science in Biotechnology.

Assoc. Prof. Dr. Gülay ÖZCENGİZ

Supervisor

Examining Committee in Charge:

Assoc. Prof. Dr. Gülay ÖZCENGİZ

Prof. Dr. Gürdal ALAEDDİNOĞLU (Co-Supervisor)

Dr. Fadil Ekrem SHARIF

Prof. Dr. Velittin GÜRGÜN

Assoc. Prof. Dr. Zeki KAYA

ABSTRACT

CHARACTERIZATION OF VARIOUS *Rhizobium cicer* STRAINS AND ASSESSMENT OF THEIR SYMBIOTIC EFFECTIVENESS

İÇGEN, Bülent
M.S. in Biotechnology
Supervisor: Assoc.Prof.Dr. Gülay ÖZCENGİZ
Co-Supervisor: Prof.Dr. Gürdal ALAEDDİNOĞLU
June, 1994, 124 Pages

Biological nitrogen fixation by the legume-*Rhizobium* symbiosis serves as a pollution-free and cheap alternative to the use of chemical fertilizers. The objective of this study was to select and characterize effective rhizobia for the improvement of chickpea yields, an economically important crop in our country, and to determine the role of various parameters on the success of symbiosis.

Two local isolates (1aa2 and 11-a) and five standard (385, 620, 3233, 3371, 3378) *Rhizobium cicer* strains were compared for their ability to nodulate chickpea and to improve the yields of this plant. Strain 385 was found to be the best as it significantly increased shoot dry weight and gave the highest number of root nodules.

The strains were characterized in terms of their antibiotic resistance and plasmid patterns as well as the production of bacteriocin. Plasmids could not be detected in most effective strain (385) while one of the nonnodulating strains (3233)

did have five or six high-molecular weight and two low-molecular weight plasmids. Strain 620 was found to harbor two plasmids of 75.8 and 46.8 Kb, respectively.

The use of mixed inocula was much better than the use of single strains, giving rise to 30 to 35 fold increases in total nitrogen. The competitiveness of strains applied in mixture was also assessed by determining both the nodule occupancy and rhizospheric colonization. Strain 620 appeared to be the most competitive organism in inducing root nodules while Y-29 was the best in terms of rhizospheric colonization.

As the inoculum size increased, the nodule number and shoot dry weight also increased. An inoculum size of 10^9 cells per seed was found to be optimum. The strains used in this study responded differently to soil pH, still pH values as low as 5.0 or as high as 9.0 did not completely inhibit nodulation. The effect of varying the concentrations of some minerals on the outcome of symbiosis was also investigated. Increased phosphate concentrations did have an adverse effect on nodulation and plant yields, and calcium effect varied depending on the inoculant strain. Ferric iron, on the other hand, was not necessary for nodulation.

The method of nodulation in agar tubes was tested in our laboratory for its feasibility. In spite of its ease as well as the economy of space and materials, the resulting symbiotic system did not give reliable results.

Key Words: *Rhizobium cicer*, Root nodulation, Symbiotic effectiveness, Competitiveness, Symbiotic nitrogen fixation.

Science Code: 614.02.07

ÖZ

ÇEŞİTLİ *Rhizobium cicer* SUŞLARININ KARAKTERİZASYONU VE SİMBİYOTİK ETKİNLİKLERİNİN DEĞERLENDİRİLMESİ

İÇGEN, Bülent
Yüksek Lisans Tezi, Biyoteknoloji Anabilim Dalı
Tez Yöneticisi : Doç.Dr. Gülay ÖZCENGİZ
Yardımcı Tez Yöneticisi : Prof. Dr. Gürdal ALAEDDİNOĞLU
Haziran, 1994, 124 sayfa

Legüm-*Rhizobium* simbiyozu ile gerçeklesen biyolojik azot fiksasyonu, kimyasal gübrelerin kullanımına temiz ve ucuz bir alternatif oluşturmaktadır.Bu çalışmada, ülkemizde ekonomik bir öneme sahip bir baklagil olan nohut bitkisinin verimliliğinin arttırılmasında kullanılabilecek etkin *Rhizobium* suşlarının seçimi, karekterizasyonu ve çeşitli parametrelerin simbiyotik ilişki üzerine etkinliklerinin belirlenmesi amaçlanmıştır.

Rhizobium cicer' in iki yerli izolatı (1aa2 ve 11-a) ve beş ayrı standart suşu (385, 620, 3233, 3371, 3378), nohutu nodule etme yetenekleri ve bitki verimliliğini arttırmadaki etkinlikleri bakımından karşılaştırılmışlardır. 385 kod nolu suş, en yüksek bitki üstü kuru ağırlığı ve en fazla sayıda kök nodülü oluşturması nedeniyle en etkin suş olarak seçilmiştir.

Rhizobium cicer suşları, antibiyotik dirençlilik kalıpları, plazmid profilleri ve bakteriosin üretimi bakımından karakterize edilmişlerdir. Etkinliği çok yüksek olan 385 suşunda hiç plazmid gözlenemezken nodülasyon yapamayan 3233 suşunda beş veya altı adet yüksek moleküler ağırlıklı, iki adet de düşük moleküler ağırlıklı plazmidin varlığı gösterilmiştir. 620 suşunun ise sırasıyla 75.8 ve 46.8 Kb büyüklüğünde iki ayrı plazmide sahip olduğu bulunmuştur.

Birden fazla suşun karışımından oluşan aşılamalar, bitkilerin toplam azot içeriklerinde 30-35 misli artışa neden olmuş, bu da karışık inokulum kullanmanın suşlarşn tekli kullanımlarından çok daha iyi sonuç verdiğini göstermiştir. Karışımlardaki suşlarin rekabet kabiliyetlerinin incelenmesi amacıyla, bu suşların hem nodüllerde, hem de rizosferde yer alma oranları karşılaştırılmıştır. 620 suşunun kök nodüllerini indüklemede en başarılı olduğu, Y-29' un ise rizosferde en kalıcı suş olduğu saptanmıştır.

Aşılama miktarındaki artışa bağlı olarak, suşların oluşturduğu nodüllerin ve bitki gövde kuru ağırlığının artış gösterdigi, optimum aşılama için ise etkin suşlar için tohum başına 10^9 hücre gerektiği sonucuna varılmıştır. Çalışmada kullanılan suşlar, toprak pH' sindaki değişimlere farklı yanıtlar göstermişler, yine de pH 5.0 veya 9.0 gibi düşük yada yüksek değerlerin nodülasyonu tamamen inhibe etmediği bulunmuştur. Bazı minerallerin simbiyotik ilişkinin sonuçları üzerine etkileri ayrıca belirlenmiştir. Yüksek fosfatın olumsuz etkisi, kalsiyumun ise inokulant suşa bağlı olarak değişen etkisi olduğu kaydedilmiştir. Diğer yandan, okside formdaki demir nodülasyon için gerekli değildir.

Agar tüplerinde nodülasyon için geliştirilmiş olan teknik, kullanılabilirliği bakımından test edilmiştir. Kolaylığı, yer ve materyal ekonomisi sağlamadaki uygunluğuna karşın, oluşturulan simbiyotik sistemin güvenilir sonuçlar vermediği görülmüştür.

Anahtar Kelimeler: *Rhizobium cicer*, Kök nodülasyonu, Simbiyotik etkinlik, Rekabet kabiliyeti, Simbiyotik azot fiksasyonu.

Bilim Dalı Sayısal Kodu : 614.02.07

ACKNOWLEDGMENTS

I would like to express my sincerest gratitude to my supervisor Assoc. Prof. Dr. Gülay ÖZCENGİZ for her invaluable guidance, continued advice and deep interest throughout this study.

I also extend my great indebtedness to Prof. Dr. Gürdal ALAEDDİNOĞLU for his invaluable suggestions and constructive criticism.

My thanks are also due to Dr. Fadil Ekrem SHARIF for his continuos help and invaluable suggestions.

I am also grateful to Prof. Dr. Velittin GÜRGÜN and Dr. Kamuran AYHAN who supplied the bacterial strains.

Further thanks are extended to my partner Esra ERDAL and to all other laboratory mates who through their friendship made life so enjoyable. I am also thankful to Alper ARSLANOĞLU and İrfan KANDEMİR for their help in typing the manuscript. My special appreciation is extended to Yavuz DARENDELİOĞLU for his kind assistance in statistical analysis.

Finally, I am grateful to my father Ali İÇGEN, my mother Mahiperiz İÇGEN, my brother Levent İÇGEN and to my friend A.Emre KARAÖZ for their support and encouragement all the time.

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

LIST OF SYMBOLS

N_2	: Nitrogen
Kb	: Kilobase
YEM	: Yeast Extract Mannitol
VAM	: Vesicular Arbuscular Mycorrhizal Associations
bv	: biovar
Chr	: Chromosomal DNA
Str	: Streptomycin
Spc	: Spectinomycin
Lin	: Lincomycin
Kan	: Kanamycin
Chl	: Chloramphenicol
Clox	: Cloxacilin
Gen	: Gentamicin
Nal	: Nalidixic acid
Tet	: Tetracyclin
Neo	: Neomycin
Ami	: Amikasin
Amp	: Ampicillin

CHAPTER 1

INTRODUCTION

Gaseous nitrogen (N_2) constitutes nearly 80 % of the atmosphere that we breathe, but it is inaccessible to humans and to all animals, plants, fungi, and virtually to all bacteria. However, nitrogen in an organic form is a major component of all living things (Figure 1.1). Proteins, nucleic acids, vitamins, and numerous other 'vital' molecules all contain this form of nitrogen. How then is the vast reservoir of inert nitrogen gas made available for the assembly of the repertoire of nitrogenous organic molecules ? The answer is biological nitrogen fixation. A relatively small of bacterial species have the special ability to reduce or 'fix' atmospheric N_2 to form ammonia, a product that can be used by plants and other microbes as a building block for the synthesis of amino acids and other nitrogenous compounds. On a global scale the amounts of nitrogen fixed by these bacteria are impressive (Long, 1989). Large amounts of nitrogen are also fixed by chemical means, during the manufacture of nitrogenous fertilizers. The manufacture of fertilizers, however, requires vast inputs of energy.

The basic commercial process for manufacturing ammonia consists of catalytically reacting hydrogen with nitrogen under high pressure and temperature (Shanmugam and Valentine, 1975):

$$3H_2 + N_2 \leftrightarrow 2NH_3$$

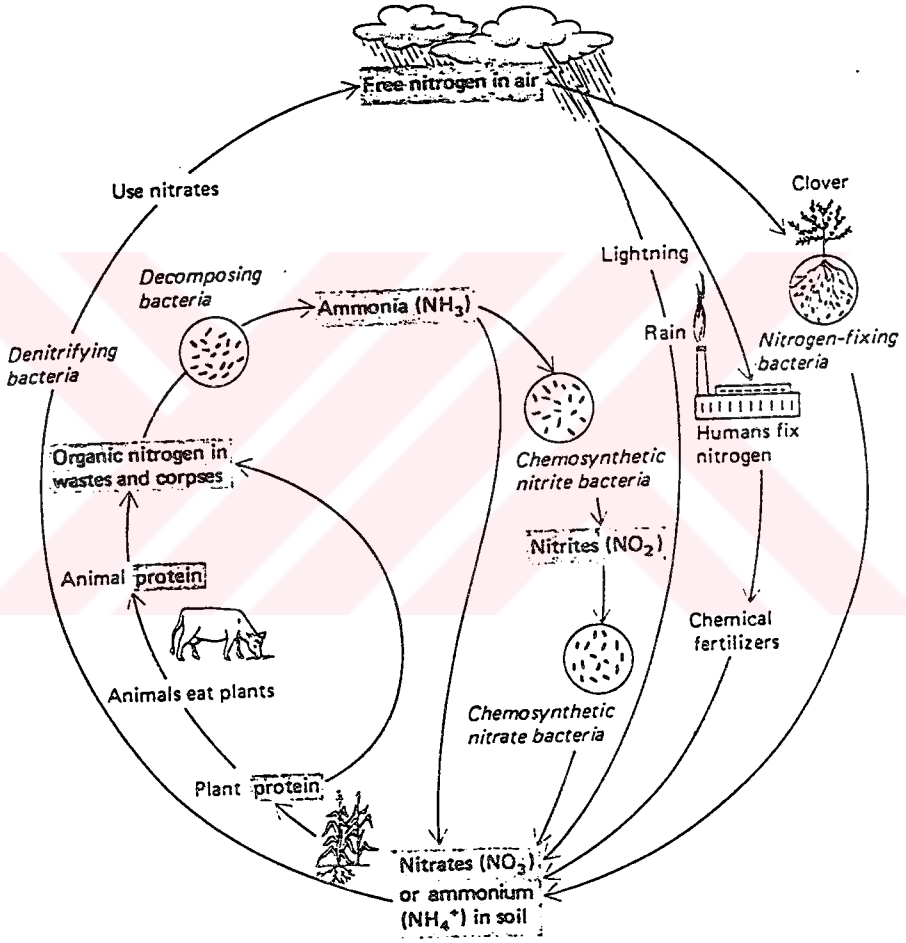


Figure 1.1. The nitrogen cycle

This process, called Haber-Bosch process, has not changed since its introduction in 1913. The dramatically rising price of petroleum as a source of hydrogen, and the many ecological and economical limitations to the heavy use of chemical fertilizers, have stimulated much interest in nitrogen fixation by microorganisms. In addition, large areas of the agricultural world are, even now, too poor to buy enough commercial nitrogen fertilizer to give optimum crop yields.

Shifting from chemically to biologically fixed nitrogen is also desirable from an environmental point of view. Much of the chemical fertilizer that is applied to crops is wasted by leaching from the soil. At worst, this can generate an acceptably high concentrations of nitrate in drinking water and can lead to the eutrophication of water systems with a consequent overgrowth of algae and other plant-life and a decline in fish and shellfish populations. The increase of nitrate ions in drinking waters and rivers also causes methaemoglobinemia (blue baby disease) in animals and infants (Alexander, 1977; Johnston, 1990).

A major long-term goal of research programs in biological nitrogen fixation is reducing the current reliance on chemical fertilizers for crop plant growth by improving the efficiencies and ranges of the nitrogen fixing organisms.

The conversion of free nitrogen to ammonia is accomplished by several species of microorganisms. These include blue green algae and several bacterial species all of which have some common features:

Firstly, all are bacteria (Table 1.1). Despite many investigations no nitrogen fixers have yet been found among the eukaryotes. If nitrogen fixing capabilities confer selective advantage to an organism, why then no nitrogen fixing yeasts or no

nitroplasts that have not yet evolved as chloroplasts and mitochondria that have taken up permanent residence in cells and established the most intimate of symbiosis? The answers to these questions are unknown. It may simply be that the ability to fix nitrogen has evolved relatively recently so that sufficient time has not elapsed for an eukaryotic nitrogen fixer to evolve.

Secondly, the members of nitrogen fixing club are taxonomically both wide-ranging and sporadic in occurrence. Representatives have been found in groups as diverse as the *Cyanobacteria* (the blue green algae), *Archaebacteria* and in both Gram (+) and Gram (-) bacteria. Lateral evolution in which the nitrogen fixation genes (*-nif* genes as they are called-) has been transferred from one bacterial type to another may be the cause of this taxonomic distribution.

Table 1.1. Nitrogen-fixing bacteria (from Johnston, 1990)

Species	Bacterial group	Comments
Klebsiella pneumoniae	Gram-negative	Model system for *nif* genetics
Azotobacter vinelandii	Gram-negative	Fixes nitrogen in air;contains a protein that protects nitrogenase from oxygen damage
Rhizobium species	Gram-negative	Fixes nitrogen in legume root nodules
Rhodospirillum, Rhodopseudomonas	Gram-negative	Purple-green photosynthetic bacteria
Frankia	Gram-positive actinomycete	Fixes nitrogen in nodules of various woody trees and shrubs
Clostridium	Gram-positive	Obligate anaerobe
Anabaena	Filamentous blue-green algae	Fixes nitrogen in specialized cells called heterocysts; some species associate with higher plants such as cycads and *Azolla*
Methanococcus	Archaebacterium	
Azospirullum	Gram-negative	Associated with roots of grasses

Thirdly, many of the bacteria do not fix nitrogen by themselves, but work in a symbiotic interaction with higher plants. The reason for this takes us to the question of energy. The triple bond that joins the two nitrogens is a tough one to break. When the bacteria associate with carbon-fixing green plants the result is nutritional trade-off. The plant gets its fixed nitrogen and the bacterium acquires the fixed carbon it needs for energy. The diversity of such symbiosis is great (Nap and Bisseling, 1990).

Finally, the special ability of nitrogen fixing bacteria to reduce nitrogen to ammonia depends on the possession of an enzyme system called the "Nitrogenase Complex". The complex appears to be very similar in all the nitrogen fixers studied to date and the information collected from one is almost certainly applicable to others.

Current knowledge indicates that the nitrogenase complex is composed of six proteins and contains two different enzyme activities (Stanley *et al.*, 1972), one called simply nitrogenase and the other called nitrogenase reductase (Figure 1.2). The nitrogenase component of the complex contains four subunits, two copies of each of two different proteins. Its structure also includes a cofactor, iron-molybdenum cofactor, which contain the metal iron and molybdenum (Hardy *et al.*, 1971). Despite many years of study, the structure of the cofactor is unknown.

The precise mechanisms on to how nitrogenase works are not completely clear. The nitrogen almost certainly binds to the cofactor after which it is reduced to ammonia (Mortenson, 1968) by the addition of electrons and hydrogen ions. The hydrogen ions are obtained from water through a number of steps the exact nature of which is still a matter to be debated. The reduction of nitrogen is energy-expensive requiring 20-30 molecules of ATP to support the reduction of one molecule of nitrogen to ammonia. Moreover, the nitrogenase reaction is inherently wasteful in that

is also reduces hydrogen ions to molecular hydrogen which is given off as a gas. Nitrogenase reductase has a molecular weight of 60.000 and consists of two identical protein subunits containing clusters of iron and sulfur. As its name suggest, the enzyme reduces nitrogenase thereby replenishing the electrons used to reduce the nitrogen. Functional hybrid molecules of nitrogenase have been constructed by mixing components prepared from different species of bacteria (Detroy *et al.*, 1968), suggesting that there is considerable chemical homology between nitrogenase enzymes in different organisms. The various partial reactions catalyzed by nitrogenase such as ATP-driven hydrogen evolution and the reduction of triple bonded compounds, acetylene, cyanide and azide are common to all nitrogenases as is inhibition by carbon monoxide (CO) (Hardy *et al.*, 1971). Nitrogenase activity is routinely assayed by gas chromatography technique which separates and detects ethylene (product) and acetylene (substrate).

A very important fact about nitrogenase is that it is deactivated by oxygen. Upon exposure to oxygen, the enzyme irreversibly loses half of its activity in about 30 seconds, a problem that seems to be common to the nitrogenases of all nitrogen-fixers. The different organisms have found varying ways for solving the oxygen problem. One strategy used by bacteria of the *Clostridium* genus, is to live in an oxygen-free environment; for these bacteria, the risks of oxygen-damage never arise. Other nitrogen-fixers , such as *Klebsiella pneumoniae,* can live either with or without oxygen, but only fix nitrogen when they are growing anaerobically (Stanley *et al.*, 1972).

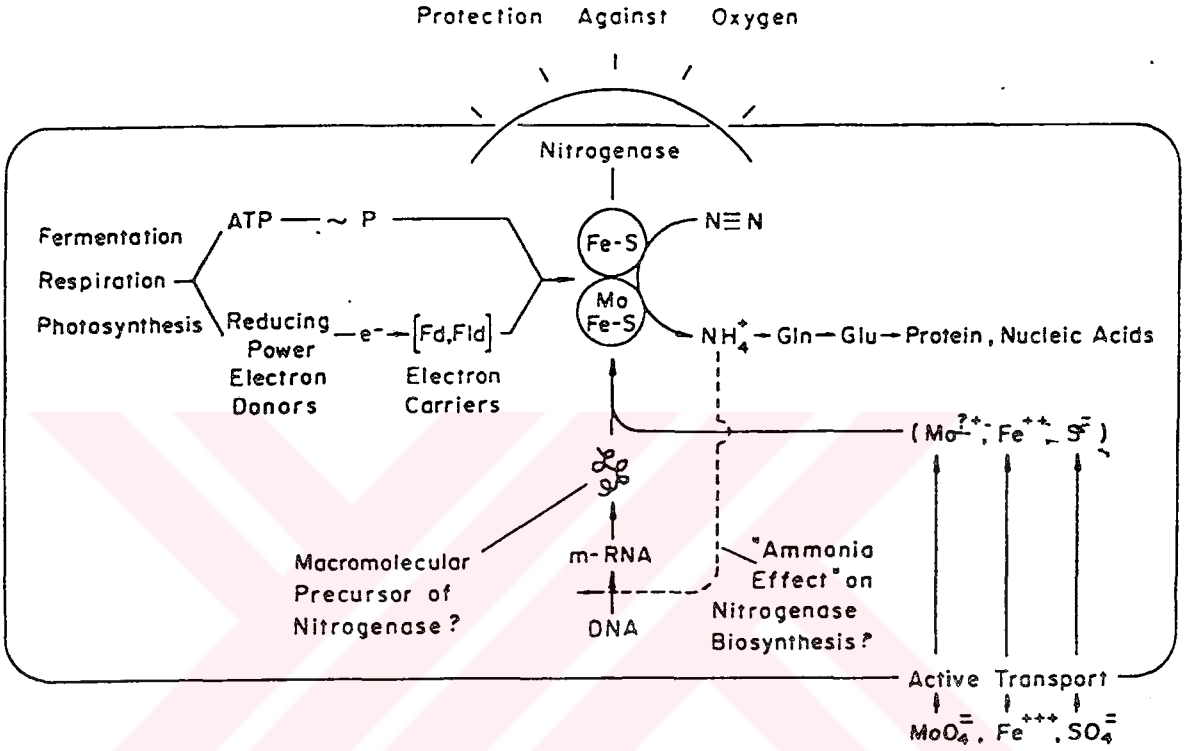


Figure 1.2. Summary of general concepts of nitrogen fixation (Stanley *et al.*, 1972). The barrier against inhibitory levels of oxygen is required in aerobic organisms.

The blue green algae, which can both carry out photosynthesis and fix nitrogen, would seem to be playing with fire because oxygen is actively liberated during photosynthesis. In some blue green algae, there is a temporal separation of nitrogen fixation, which occurs at night, and photosynthesis, which takes place during the day. Various filamentous blue-green algae, such as *Anabaena,* have developed more sophisticated solution. The cells of the filaments are generally photosynthetic, but under conditions that favour nitrogen fixation some of them differentiate to produce morphologically distinct non-photosynthetic cells called "heterocysts" that fix nitrogen (Sprent, 1986).

The other strategy which is common among symbiotic nitrogen fixers is leghemoglobin. Leghemoglobin is an oxygen binding nodulin protein found in the host cytoplasm of legume nodules. The presence of leghemoglobin in the nodule is essential for nitrogen fixation. Nitrogenase is oxygen sensitive and leghemoglobin binds oxygen to reduce the oxygen tension in the nodule. Leghemoglobin releases oxygen to metabolically-active bacteroids at a concentration that will not damage the nitrogenase complex but will still allow bacteroid respiration. The globin apoprotein is synthesized by the legume host and is specifically induced on infection of plant by the bacteria. The heme portion of leghemoglobin is synthesized by bacteria. Heme biosynthesis in the nodule is due to the microaerophilic conditions that one would expect to occur in a nodule and not in response to a specific signal molecule. Furthermore, mutants defective in heme are able to induce leghemoglobin synthesis. This demonstrates that the heme portion of leghemoglobin does not specifically induce the synthesis of the globin apoprotein. It is not known whether rhizobia constitutively produce the signal molecule which induces leghemoglobin mRNA synthesis or whether the signal molecule is made in response to a certain stage of infection (Long, 1989).

An increasing number of nitrogen fixing bacteria is studied with the techniques of genetics and molecular biology. The most plant-interacting microbes not only exploit plants as a nutritional source but, over evolutionary time, cause less and less damage to their host (Djordjevic *et al.*, 1987). Plant simbionts provide good examples of primitive, intermediate, and advanced stages of selective evolution, which may be reflected in *Rhizobium*-plant interactions (Gabriel, 1986; Hodgsen and Stacey, 1986; Vincent, 1980). The number of microbial and plant genes involved in the interactions increases overtime and also changes with the strategy used for niche occupation. The most advanced symbionts appear to exhibit the highest degree of host selectivity and mutual recognition. *Rhizobium* bacteria stimulate leguminous plants to develop root nodules, which the bacteria infect and inhabit. Ultimately, the two organisms establish metabolic cooperation: the bacteria reduce (fix) molecular nitrogen to ammonia, which they export to the plant for assimilation; the plant reduces carbondioxide to sugars during photosynthesis and translocates these to the root where the bacteria use them as fuel (Long, 1989; Appelbaum, 1990).

The plant family *Leguminosae (Fabaceae)* is the third largest family in the *Angiosperms*, spreads from the tropics to arctic regions, and includes forms varying from annual herbs to large trees. It doubtlessly owes at least some of this diversity and success to its ability to grow independently of often scarce soil nitrogen. Only one non-legume plant, *Parasponia* , has been found to form symbiotic root nodules with *Rhizobium.* The question of what makes the legumes unique is an important and provoking one. There is also considerable specificity of individual strains or species of *Rhizobium* for particular groups of plants (Sharon, 1989). The ecological and economical importance of nitrogen fixation has justly earned research attention for the *Rhizobium*-legume symbiosis. The system has an additional, fundamental attraction. During a complex series of developmental steps, the bacteria and the plant each

influences the other in fundamental activities such as cell division, gene expression, metabolic function, and cell morphogenesis. Analysis of bacterial influence on these processes may lead to identification of otherwise elusive components that are parts of the indigenous plant systems for signal transduction, gene regulation, cell division and cell wall formation.

Rhizobia are Gram (-) soil bacteria capable of inducing nitrogen fixing nodules on specific legumes and have been classified into two genera ; the fast growing *Rhizobium* species and the slow growing *Bradyrhizobium*. *Rhizobium* infection is normally (but not always) restricted to specific plant species (Djordjevic *et al.*, 1987). Various studies on the host range of *Rhizobium* have identified the following cross-inoculation groups:(Table 1.2) *R. trifoli*-clovers, *R. leguminosarum* -peas and lentils, *R. phaseoli* -beans, *R. meliloti*- alfalfa, *R. fredii* and *Bradyrhizobium japonicum* -soybean (Halverson and Stacey, 1986; Vincent, 1980).

Although rhizobia are classified as a subgroup of the *Rhizobiaceae* (which includes the well-known plant pathogen *Agrobacterium*), they are infrequently considered as plant-pathogenic or parasitic bacteria (Hodgsen and Stacey, 1986; Halverson and Stacey, 1986 ; Bauer, 1981; Beringer *et al.*, 1980). Rhizobia are somewhat unique among soil microorganisms in their ability to form nitrogen-fixing symbiosis with legumes. To enjoy the benefits of this partnership, however, the rhizobia must not only exhibit saprophytic competence among other soil microorganisms but must also compete other rhizobia for infection sites on legume roots. Potential for physiological versatility is therefore an important trait contributing to their adaptation to the competitive and complex soil environment.

Table 1.2 *Rhizobium* species and their hosts (from Jonston, 1990)

Bacterial species	Host plant	Comments
R. leguminosarum *R. trifolii* *R. phaseoli*	Peas, *Vicia* (Broadbean) lentils Clover *Phaseolus* bean	Species are very closely related to each other
R. loti	Lotus	Lotus is also nodulated by *Bradyrhizobium*
R. lupinii	Lupin	-
R. meliloti	Alfalfa	-
R. sesbania	Sesbania	Induces stem and root nodules on *Sesbania*. Also fixes nitrogen in free living culture
R. fredii	Soybean	Induces non-fixing nodules on most soybean cultures
B. japonicum	Soybean	Some strains fix nitrogen in free living culture
B. 'cowpea miscellany'	Nodulates several tropical legumes cowpea and also the non-legume *Parasponium*	Has the ability to nodulate non-legume host

Strains of *Rhizobium* can be described according to their growth in solid and liquid media. The size, shape, color and texture of colonies and the ability to alter pH of the medium are generally stable characteristics useful in defining strains or isolates (Somasegaran and Hoben, 1985). When grown on standard yeast extract mannitol agar medium (YEM), colonial shape is usually discrete; round colonies, varying from flat to domed and even conical shape appear on agar surface. Colonies usually have smooth edges. When grown subsurface in the agar, they are typically lens-shaped, their colour changing from white opaque to milky or translucent. The opaque colonial growth is usually firm with little gum, whereas less dense colonies are often gummy and soft. Colonies may be glistening or dull, evenly opaque or translucent. Growth generally takes 3 to 5 days for fast-growers (*R. leguminosarum*,

R. phaseoli), 5 to 7 days for slow growers (*Bradyrhizobium japonicum*) . Growth rate varies with the incubation temperature (optimum temperature 26 to 30°C), origin of culture, aeration (in liquid culture) and composition of medium. When well-separated on agar plates, colony size may vary from 1 mm for many slow-growing strains to 4-5 mm for fast-growing strains. The indicators and selective media are used for rhizobia. They generally do not absorb Congo Red when plates are incubated in the dark and colonies remain white, opaque or occasionally pink. Contaminant organisms usually absorb the red dye. However reactions depend on the concentration of Congo Red and age of the culture. Rhizobia will absorb the red dye if plates are exposed to light for an hour or more after growth has occurred or during the incubation. Freshly prepared Yeast Extract Mannitol (YEM) agar plates containing bromthymol blue have a pH of 6.8 and are green. Slow growing rhizobia show an alkaline reaction in this medium, turning the dye blue. Fast growing rhizobia show an acidic reaction turning the medium yellow.

The interaction of *Rhizobium* and *Bradyrhizobium* with roots of leguminous plants results in the development of root nodule. Within this structure, the bacteria reduce atmospheric nitrogen to ammonia, which is then internally assimilated by the plant (Caetano-Anolle's *et. al.*, 1991).

Signal exchange between the plant and the bacterium is evident throughout the nodulation process and several of the signal molecules have been identified recently. Rhizobia exist as soil saprophytes until they contact and colonize around root surface of an appropriate legume plant. Recognizable steps in the early infection process are summarized in Figure 1.3 and Figure 1.4 (Djordjevic and Weinman, 1991; Denarie *et al.*, 1992). Specific factors released from the plant root tissue are known to induce the expression of *Rhizobium* nodulation (*nod*) and host specific

nodulation genes. These genes are specifically required for the infection, colonization and establishment (initially) of a parasitic life style. The *nod* genes are located on large plasmids (mega plasmids) which are called as *Sym* plasmids.

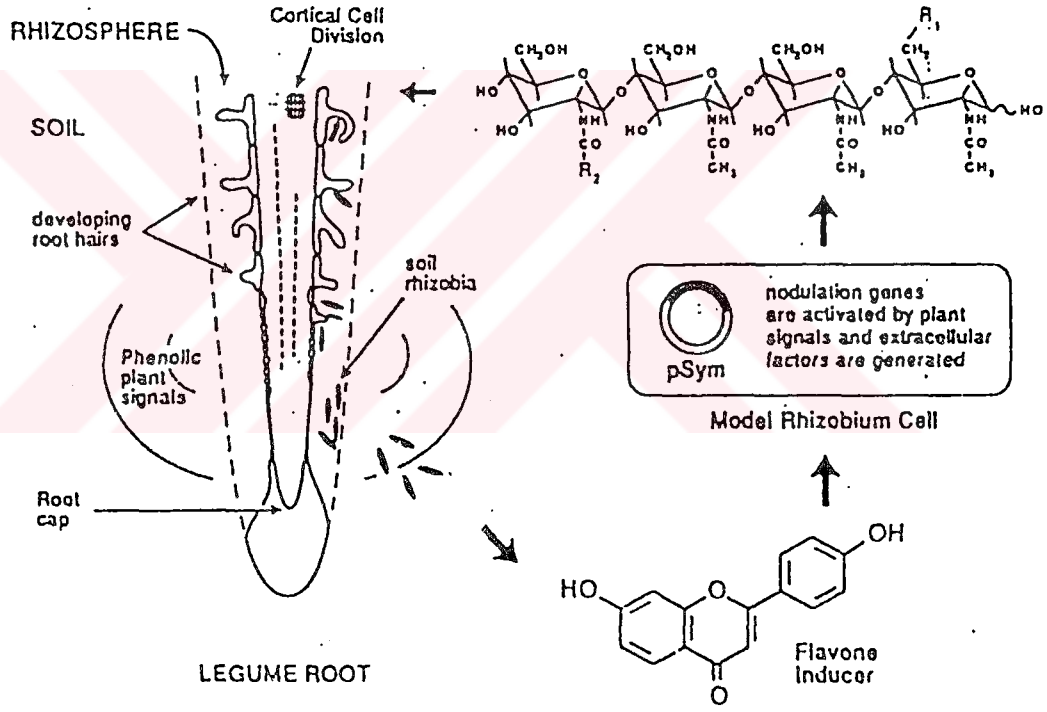


Figure 1.3. Signal exchange between the legume root and an invading *Rhizobium* during the preinfection stage (Djordjevic and Weinman, 1991).

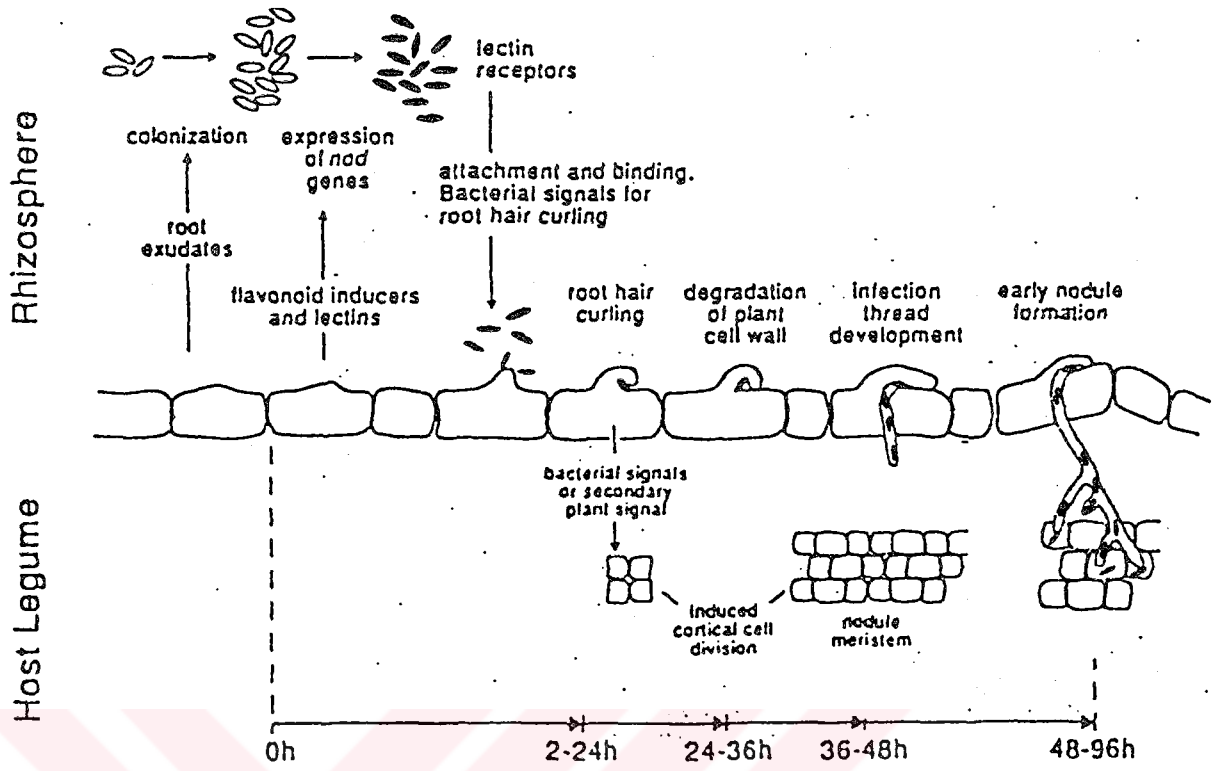


Figure 1.4. Summary of the early infection events evident during Rhizobium legume interactions (Djordjevic and Weinman, 1991).

Rhizobia colonize the root surface and attach to epidermal cells (including root-hair cells) and remain in a commensal association, inducing little or no visible effect. Mature root hairs are rarely infected. Flavonoid compounds which are needed to stimulate the expression of the *Rhizobium* nodulation genes, are secreted in high concentrations, which coincide with location of the emerging root hairs. Rhizobia respond by releasing unknown low molecular weight factors that can cause marked root hair distortion and curling or that induce cortical cells to undergo repeated cell division. There is evidence that attachment to the root hair cells is mediated by plant-secreted lectins. It mediates specific attachment or some other important recognition event during infection (Djordevic and Weinman, 1991). Bacteria that become

entrapped in the folds of the curled root hair rapidly degrade the nearby root hair wall and colonize this area. The nucleus of the root-hair cell migrates to this infection site and synthesis of new cell wall material begins. This migration maybe a repair response localized to the plant cell wall. The new wall continues to develop around the penetration site ahead of the bacteria. This wall formation results in the production of an infection thread (host material) in which the rhizobia divide and proliferate. As a result of these events cortical cell in the root are stimulated to divide and eventually form the nodule tissue. During the release of the bacteria into the nodule they are surrounded by a host derived peribacteroid membrane (Figure 1.5). The encaptured bacteria ultimately differentiate into bacteroids, which are able to fix nitrogen in a form that the plant can assimilate. While in the infection thread, rhizobia are parasites; they may switch to mutualistic symbionts if a nitrogen fixing response occurs (Figure 1.6).

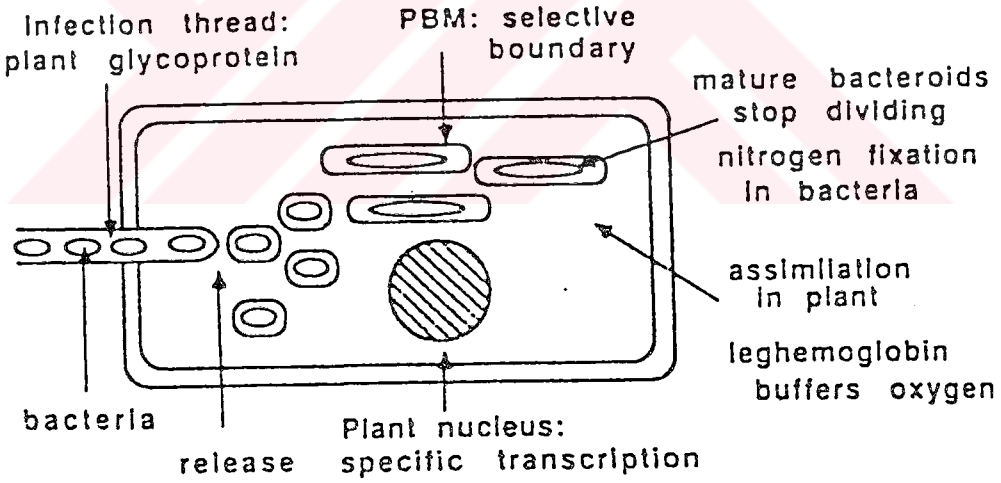


Figure 1.5. Components of the functioning symbiotic cell: Bacteria are released into the plant cell by endocytosis with plant plasma membrane (Long, 1989).

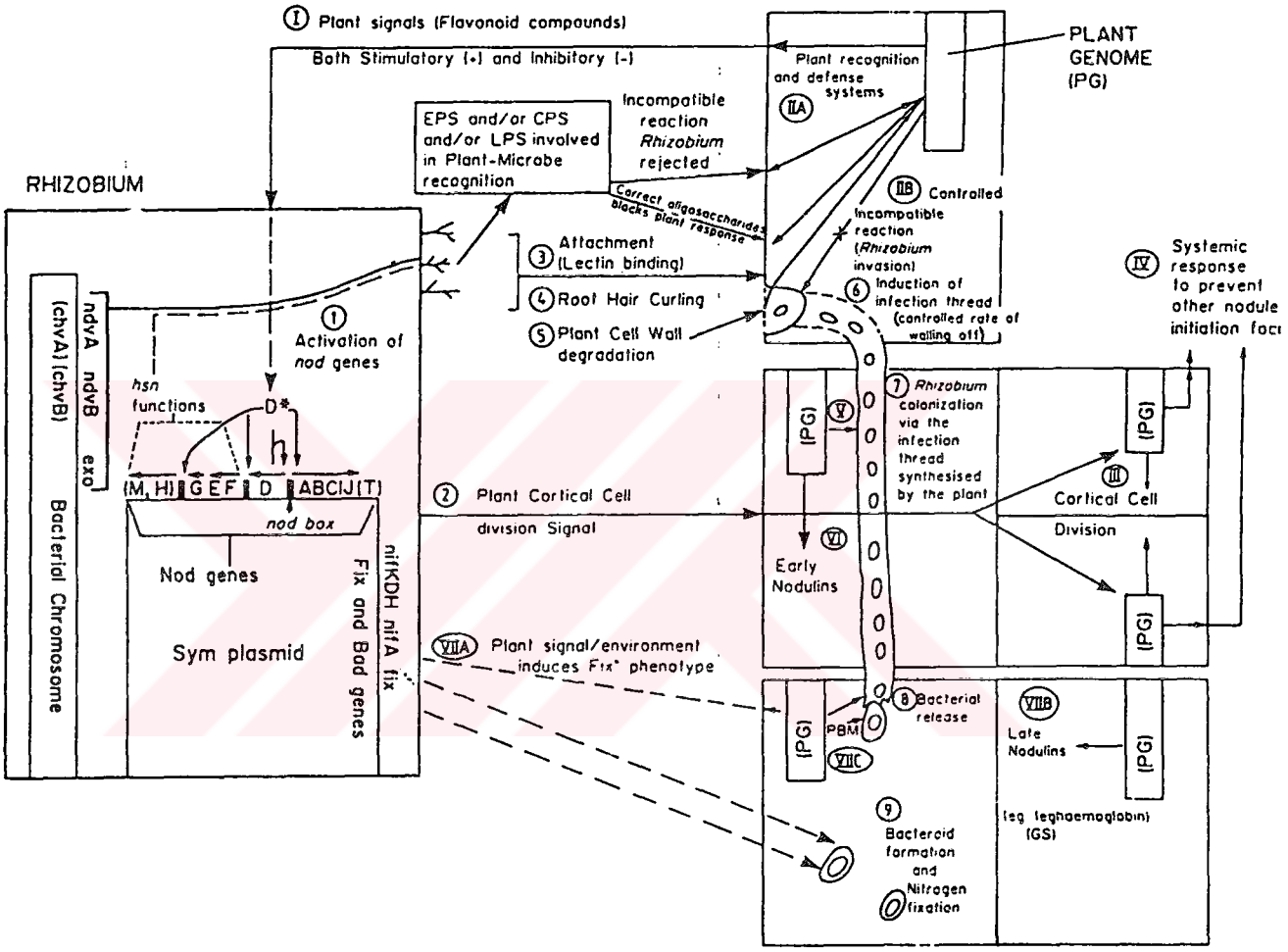


Figure 1.6. Schematic representation of the interaction and various steps during the infection of legume root cells by *Rhizobium* bacteria (Djordjevic *et al.*, 1987).

Competition for nodulation is usually measured by comparing the ability of introduced *Rhizobium* strains to form nodules on the chosen host. Many nodules are produced by mixed infections (Broughton, 1978; May and Bohlool, 1983) and upon decay, the ratios of inhabitants within these nodules will be the numbers of the competitive strains in soil. Furthermore, as rhizobia have the capacity to establish themselves in the absence of the host legume (so-called saprophytic competence), their abundance in the rhizospheres of nonhosts or in bare soil will also influence later inoculations.

Ecological factors affect competition both directly through their effect on rhizobia and indirectly through their effect on plant growth. Quite obviously, environmental factors affect the growth of both plant and bacteria (Figure 1.7). As plants must develop in rhizosphere to support rhizobial growth as well as to build a morphologically developed nodule to house the invading rhizobia, we may assume that any factor that adversely affects plant growth will also profoundly affect competition for nodulation (Dowling and Broughton, 1986). Environmental factors which directly influence competition can be listed as follows:

Soil type: Soil is a reservoir of *Rhizobium* strains and the intrinsic make-up of soil can affect the outcome of competition. Bowen and Rovira (1976) showed that the growth rate of *Rhizobium* in soil without plants is slow; a generation time of 200 hr was estimated, as opposed to 12 hrs in the rhizosphere of legumes. Similarly, no increase in size of *B. japonicum* inocula was observed in fallow soil, while 100-fold increase was noted in the rhizosphere of Soybean (Pena-Cabriales and Alexander, 1983 a or b). Although *Rhizobium* numbers tend to be low in soil without legumes, the native rhizobia are well-adapted to living as soil saprophites

(Damirgi *et al.*, 1967; Ham *et al.*, 1971). The ecology of *Rhizobium* within the soil environment has been extensively reviewed (Bushby, 1982).

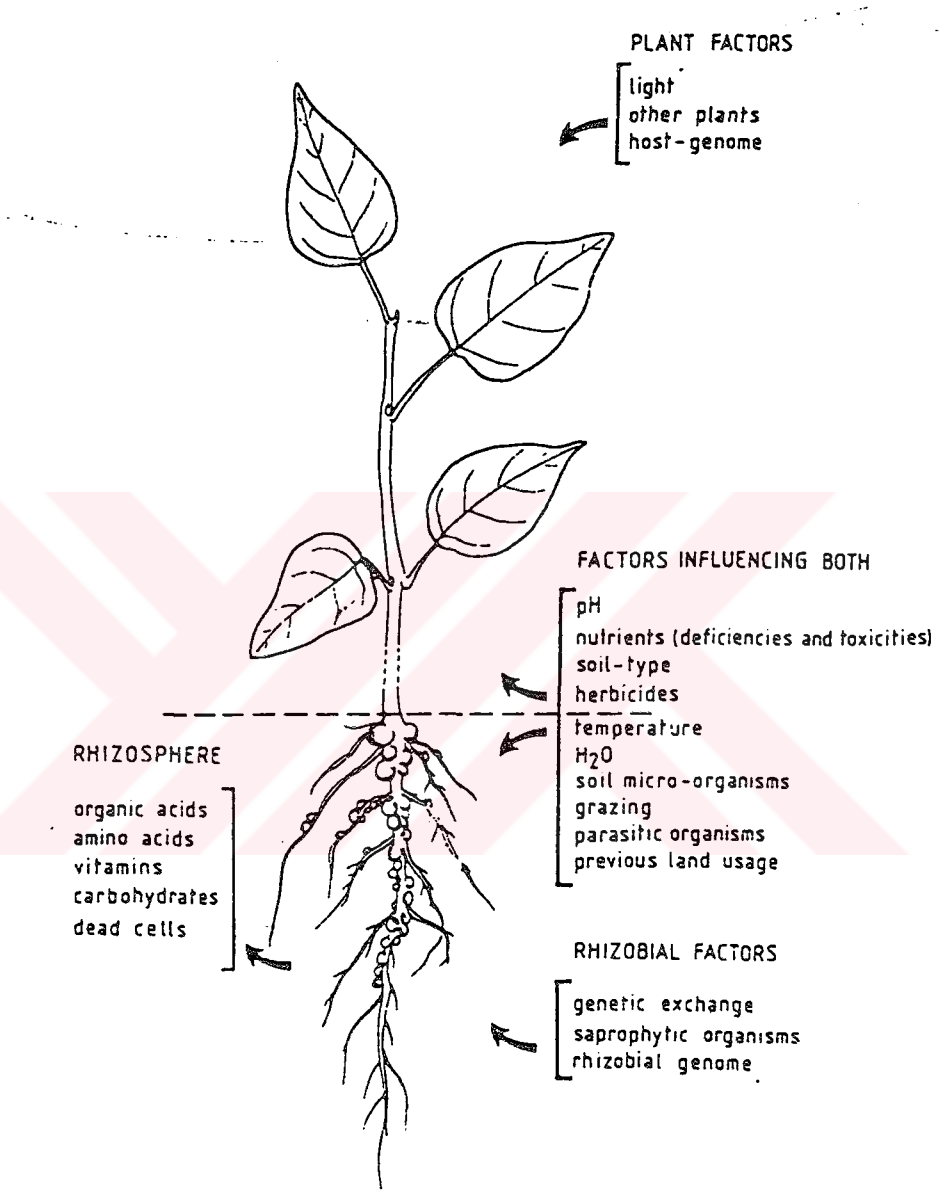


Figure 1.7. Factors that may influence the outcome of competition among *Rhizobium* strains for nodulation of legumes (Dowling and Broughton, 1986).

Combined nitrogen. (nitrate in particular): Combined nitrogen delays nodule formation (Gibson and Nutman, 1960). Herridge *et al.*, (1984) grew soybean in a field with high nitrate levels and inoculated them with an effective *B. japonicum* strain. Initiation of nodules was delayed. Nodulation occurred between 42 and 62 days after sowing, coinciding with a depletion of nitrate from the top of the soil profile. *Rhizobium* strains do not respond equally to the application of nitrogen. For example Mc Neil (1982) found that one strain of *B. japonicum* outcompeted another for nodulation sites in the presence of nitrate. In soil with relatively high nitrogen levels, this strain would have an advantage. In contrast, the addition of nitrate did not alter the outcome of the competition system with two serogroups of *B. japonicum* (Kosslak and Bohlool, 1985).

Phosphates: Phosphates are limiting factors for plant growth in acidic tropical soils. Growth of *Rhizobium* in pure cultures under low phosphate levels has been found to vary among strains (Beck and Munns,1984). A study on competition of two serogroups of *R. trifolii* demonstrated that when the soil was limed (to increase the available phosphates) the dominant serogroup was replaced by a minor one. The addition of phosphate alone had little effect on the outcome of competition between the two strains; however, the addition of phosphate and lime restored the dominance of the original dominant serogroup (Almendras and Bottomley, 1985). This suggests that phosphate limitation is exacerbated by a low pH and that the combination of pH and phosphorous levels can have a strong influence on competition for nodulation.

pH: The effect of soil pH on the survival and competition of *Rhizobium* has been well documented. Extreme soil acidity is usually linked with high aluminum levels that inhibit growth of the legume host (Franco and Munns, 1982), but it has a little effect on the survival of rhizobia in the soil (Hartel and Alexander, 1983). In

Leonard jar experiments, low pH (in liquid medium) inhibits nodulation by *R. leguminosarum, R. phaseoli* and *Bradyrhizobium spp.* (Franco and Munns, 1982; Evans *et al.*, 1980; Hohenberg and Munns, 1984). *Rhizobium* strains that are pH-sensitive also nodulate more slowly than acid-tolerant strains (Franco and Munns, 1982; Thornten and Davey, 1983). *Rhizobium* strains vary in their acid tolerance (Ayanaba *et al.*, 1983; Copper, 1982; Lowendorf and Alexander, 1983), and many fast growing rhizobia produce acids from sugars when grown in culture medium (Bromfield and Jones, 1980; Jones and Burrow, 1969; Tan and Broughton, 1981). Caetano-Anolles *et al.* (1985) suggested that the survival of rhizobia in acid soils is inversely related to the amount of acid produced, i.e. those rhizobia produce the least acid should survive in acid soils. However, acid production depends on the available substrate, even among slow-growing *Rhizobia* which are normally considered to increase the pH of the medium (Franco and Munns, 1982; Tan and Broughton, 1981). Bromfield and Jones (1980) noted a positive correlation between final pH of the culture medium and symbiotic effectiveness, but no correlation between the pH of the medium and the soil from which the isolates originated.

Soil pH can be increased by liming and this may improve nodulation and nitrogen fixation of legumes (Bromfield and Jones, 1980a). However, in an acid soil in Nigeria uninoculated soybeans produced few nodules and the addition of lime did not enhance nodulation. Inoculated plants produced effective nodules, but nodulation was not significantly improved by subsequent liming (Bromfield and Ayanaba, 1980). Damirgi *et al.* (1967) showed that *B. japonicum* serogroup 123 formed the majority of nodules on *Glycine max.* at low pH (5.9), whereas at a higher pH (8.3) another serogroup dominated. Changes in soil pH may also alter the relative proportion of nodules formed by competing strains of *R. meliloti* (Dughri and Bottomley, 1983, 1984; Rensberg *et al.*, 1982). Dughri and Bottomley (1983,

1984) were able to alter the outcome of competition between indigenous rhizobia in the soil by changing the pH. Manipulation of the pH may not always be appropriate, however. When Russel and Jones (1975) adjusted soil pH from acid to neutral / alkaline by liming, they inadvertently enhanced nodule formation by the ineffective strain. The effective strain was more competitive under acid conditions.

Temperature: Soil temperature is a major factor in the ability of *Rhizobium* to persist and compete. Strains differ in their ability to survive and grow over a range of temperatures (Hartel and Alexander,1984; Boonkerd and Weaver, 1982). The failure of *R. trifolii* inoculants to persist through more than one growing season in Western Australian soils has been attributed to the high soil temperatures in summer (Chatel *et al.*, 1973). Temperature can alter the outcome of competition between strains. Weber and Miller (1972) planted soybeans in pots containing soil with a large population of indigenous rhizobia. Different serogroups predominated in the nodules of plants that were incubated at different temperatures. A promiscuously nodulating cultivate of *G. max.* (cv. Malayan) could be nodulated by strains of *Vigna rhizobia* as well as *B. japonicum.* In mixed inoculations, the *Vigna rhizobia* were more competitive at higher temperatures (36°C) than the *B. japonicum* strain, which competed better at low temperatures (24-30°C) (Roughley *et al.*, 1980). Two strains of *R. trifolii* were inoculated onto clover plants; one strain formed the majority of nodules at 12°C, but the other strain was more competitive at 25°C (Hardarson and Jones, 1979). Obviously, temperatures also influences the optimum rate of nitrogen fixation within the root nodule, depending on the *Rhizobium* strain used. *Lotus* nodulated by a fast growing *Rhizobium* was found to fix nitrogen optimally at a lower temperature (12°C) than *Lotus* nodulated by a slow growing *Rhizobium* (Pankhurst and Layzell., 1984). So, even though temperature cannot easily be controlled under field conditions, rhizobia can be selected for optimum nitrogen

fixation or competitive abilities under the prevailing conditions. Furthermore, rhizobia with a metabolism geared to a particular temperature may compete better at that temperature.

Soil moisture: Drought conditions, which are often found in conjunction with salt stress, also affect *Rhizobium* survival and competition (Joshi *et al.*, 1981; Osa-Afiana and Alexander, 1982). *Bradyrhizobium* strains isolated from arid areas were found to be more tolerant of desiccation and high temperature than strains isolated from cooler, wetter regions (Hartel and Alexander, 1984). A study on the survival of two *Vigna* strains and two *Arachis* strains under different soil moisture and heat conditions revealed the highest mortality when high temperatures were combined with extremes of high or low moisture (Boonkerd and Weaver, 1982). Numbers of *B. japonicum* and *R. leguminosarum* measured at intervals between one and seven weeks after inoculation were lowest under extreme drought conditions (-15 bar) and were also influenced by soil type (Mahler and Wollum, 1981).

Salinity: The effect of high NaCl levels on competition is poorly understood. High salt levels alter the metabolism of rhizobia, leading to increased levels of intracellular glutamate (possibly to maintain the ionic balance between the cell and the medium) (Yap and Lim, 1983). *Rhizobium* strains isolated from nodules of *Lens* and *Pisum* to be more salt-tolerant than others. Accordingly, salt stress, altered nitrogen fixation and glutamine synthesis. A *R. meliloti* strain that was tolerant to 1.2 % NaCl was isolated (Doura *et al.*, 1984); it could nodulate *M. sativa* and fix nitrogen under salt stress conditions. However, as very low levels of salt have been found to inhibit nodule initiation in soybean split root experiments (Singleton, 1983), it seems that legumes are more sensitive than rhizobia to saline conditions.

Therefore, the role of salinity in competition among *Rhizobium* strains may be limited.

There are also some biological factors:

Bacteriophage effect: Schwinghamer and Brockwell (1978) showed that lysogenic strains of *R. trifolii* grown in sterile broth and peat culture strongly suppressed the growth of sensitive strains. There are many reports of pleiotropic effects on symbiotic ability due to phage resistance or sensitivity (Ali *et al.*, 1980; Stacy *et al.*, 1984). This influence may be due to the fact that there is a common site of interaction between phage and bacterium and between bacterium and plant, possibly the lipopolysaccharide present in the external layer of the cell (Zajac and Lorkiewcz, 1983).

Bdellovibrio: Being an intracellular parasite of *Rhizobium* (Stolp, 1973), is widespread in the soil and in the laboratory is capable of infecting and lysing large numbers of rhizobia (Parker and Grove, 1970). Although high numbers of rhizobia in the soil lead to population increases in *Bdellovibrio* (Keya and Alexander, 1975), this parasite seems to have little influence on competition and nodulation of rhizobia in the soil (Alexander, 1977).

Protozoa: Predatory protozoa have been shown to contribute to the decrease of *Rhizobium* populations in the soil (Danso *et al.*, 1975). The use of pesticides that suppress protozoa (e.g. thiram) have increased the colonization of *Phaseolus vulgaris* by *R. phaseoli* (Ramirez and Alexander, 1980).

Epiphytic bacteria: The epiphytic bacterium *Erwinia herbicola* is commonly found on the seeds of *M. sativa*. A mutant of *R. meliloti* that nodulated slowly in the presence of *E. herbicola* was found to nodulate as quickly as the wild-type strain when the bacterium was not present (Handelsman and Brill, 1985). The authors suggested that *E. herbicola* may suppress initial nodulation of this *R. meliloti* mutant by producing a toxin or by blocking rhizobial attachment sites on *M. sativa* root hair. Such interactions with epiphytic bacteria may be common in the soil and may affect competition.

Mycorrhizal associations: Legumes commonly interact with the fungi *Acaulospora, Gigaspora, Glomus* and *Sclerocytis* to produce vesicular arbuscular mycorrhizal (VAM) associations. VAM are obligate symbionts that provide usable phosphates to the plant in return for fixed carbon. In all cases examined, the legume - *Rhizobium* - mycorrhiza association is more efficient than the association of any two of these alone (Barea and Azcon-Aguilar, 1983). A study on carbon distribution in the *Vicia faba* symbiosis revealed that nodules on plants infected with VAM receive twice as much fixed carbon as those on uninfected plants (Kacely and Paul, 1982). As yet, there is no evidence that mycorrhiza influence competition among *Rhizobium* strains (Kosslak and Bohlool, 1985) or compete for *Rhizobium* infection sites (Barea and Zcon-Aguilar, 1983).

Bacteriocins: Bacteriocins are functionally defined as specific, non-self propagating agents produced by one bacterium and antagonistic toward other similar bacteria. Bacteriocin-producing strains are common among the *Rhizobiaceae* (Schwinghamer *et al.*, 1963). Two major classes of bacteriogenic substances in *Rhizobium* are recognized: low-molecular weight nonphage-like substances that diffuse readily in agar, which can be further subdivided into two types based on

tolerance to pH and membrane-binding properties (Schwinghamer, 1975); and less diffusable, high molecular weight proteins that resemble bacteriphage tail fibers. Two types of bacteriocins, small and medium (characterized according to the size of the lysis zone around the indicator bacteria), were found in field isolates of *R. leguminosarium* (Hirsh, 1978). The genes encoding medium bacteriocin production were present on large plasmids, one of which also carried *nod* genes (Johnston *et al.*, 1978).

Besides environmental and biological factors, there are also some other factors:

Herbicides. pesticides and fungicides: Being often used in agriculture, they may influence the outcome of inoculation. For example, herbicides delay nodulation in *Pisum sativum* (Magu and Bhowmik, 1984) but nodulation may resume during the later phases of plant growth. Kao and Wang (1981) found that nodulation was delayed due to plant damage. They concluded that herbicides affect the plant directly and has no influence on the survival of rhizobia. *Rhizobium* spp. have been shown to be sensitive to low concentrations of fungicides (Gillberg, 1971; Makawi and Abdel-Ghaffar, 1970), and fungicide treatment of seeds often leads to poor nodulation (Curley and Burton, 1975; Graham *et al.*, 1980). As different levels of resistance to fungicides have been observed among strains of *Rhizobium* (Afifi *et al.*, 1969; Faizah *et al.*, 1980), it should be possible to use resistant isolates as inoculants.

Effect of the rhizosphere: The rhizosphere is that portion of the soil under the direct influence of the plant root system. It is a zone rich in nutrients (particularly carbohydrates, amino acids and vitamins) for which populations of microorganisms including rhizobia compete.

Also in Turkey some researchers have investigated several aspects of Legume-*Rhizobium* symbiosis. As reviewed by Gürgün (1983), many of investigations in Turkey have been realized on the isolation of effective rhizobia and their field application. Şahinkaya (1964) chosed 8 effective strains out of 90 isolates from bean nodules and recommended them for inoculant production. Gürbüzer (1973), in another work, reported on the selection of effective Rhizobia for lentil. Göktan (1974) isolated 48 *R.phaseoli* strains and tested them by estimating dry matter and nitrogen content of the infected plants. Göktan and Madanoğlu (1975) have conducted a similar study on rhizobia-alfalfa symbiosis. Kitiş (1976) has studied on the isolation of the root nodule bacteria specific for lentil and their characteristics including nitrogen fixing ability. Çakmakçı (1977) has compared the nitrogen fixation ability of Rhizobium strains via gas chromatographic analysis. The cultural characteristics of chickpea Rhizobia and their nitrogen fixing ability as well as their competition behaviour were studied by Gürgün (1978). The strains were identified in terms of growth and acid production on peptone-glucose agar medium color of colonies and slime formation on YEM agar, gram reaction and dye absorption on YEM agar containing the indicator Congo Red. According to the results of these tests, 25 native and 10 foreign strains were kept as test organisms for further experiments. Ülgen (1978) has selected 7 effective ones out of 30 different strains isolated from Central Anatolian Plateau and its vicinity. Haktanır (1980) determined effectiveness and the nodulation competitiveness of some native and foreign *R.leguminosarum* strains in different soil groups in Turkey. The grain yield as well as the protein and oil content of soybeans and beans as affected by the use of inoculants were studied in field conditions by Gürgün (1981). Competition patterns amongst strains of *Rhizobium* can be modified by the soil type as demonstrated by Somasegaran *et al.* (1988). They also showed that competition for nodulation in

chickpea was influenced by the growth medium, chickpea genotype, and strain differences, but not the inoculation rate.

Several types of genes are involved in the induction of nodulation. The nodulation (*nod*) genes are genes required for the parasitism, infection and nodule initiation. A cluster of nodulation genes required for root-hair curling, the stimulation of infection-thread synthesis and host specific nodule induction is located on large indigenous plasmids called *Sym* plasmids in most fast growing *Rhizobium* strains (Beringer *et al.*, 1980; Banfalvi *et al.*, 1981; Brewin *et al.*, 1980; Djordjevic *et al.*, 1983; Rosenberg *et al.*, 1981). *Sym* plasmids also contain many genes required for the fixation of nitrogen (*fix* genes) and nitrogenase (*nif* genes) (Beringer *et al.*, 1980; Banfalvi *et al.*, 1981; Rosenberg *et al.*, 1981). In *Bradyrhizobium* functional analogous of these *nod*, *fix* and *nif* genes are located on the chromosome (Lamb and Hennecke, 1986; Russell *et al.*, 1985; Scott, 1986; Stacey *et al.*, 1982). Genes involved in the production of exopolysaccharides are also normally required for nodulation (Demont *et al.*, 1993; Breedveld *et al.*, 1993). Exopolysaccharide deficient mutants (*Exo*$^-$) are usually blocked early in the infection process, although nodule induction still occurs (Chakravorty *et al.*, 1982; Chen *et al.*, 1985; Finan *et al.*, 1985; Finan *et al.*, 1986; Hynes *et al.*, 1986; Leigh *et al.*, 1985).

The fast growing *Rhizobium* spp. typically have large plasmids (*Sym*) of about 200-300 kilobases (Kb) in *R. leguminosarum* and "megaplasmids" (1200-1500 Kb) of *R. meliloti* (Sharon, 1989).

The rapid advance in the analysis of the genes required for infection by *Rhizobium* spp. has facilitated the subdivision of the various genes into different

potential functional groups as presented in Figure 1.8 (Djordjevic *et al.*, 1987; Kondorosi *et al.*, 1991).

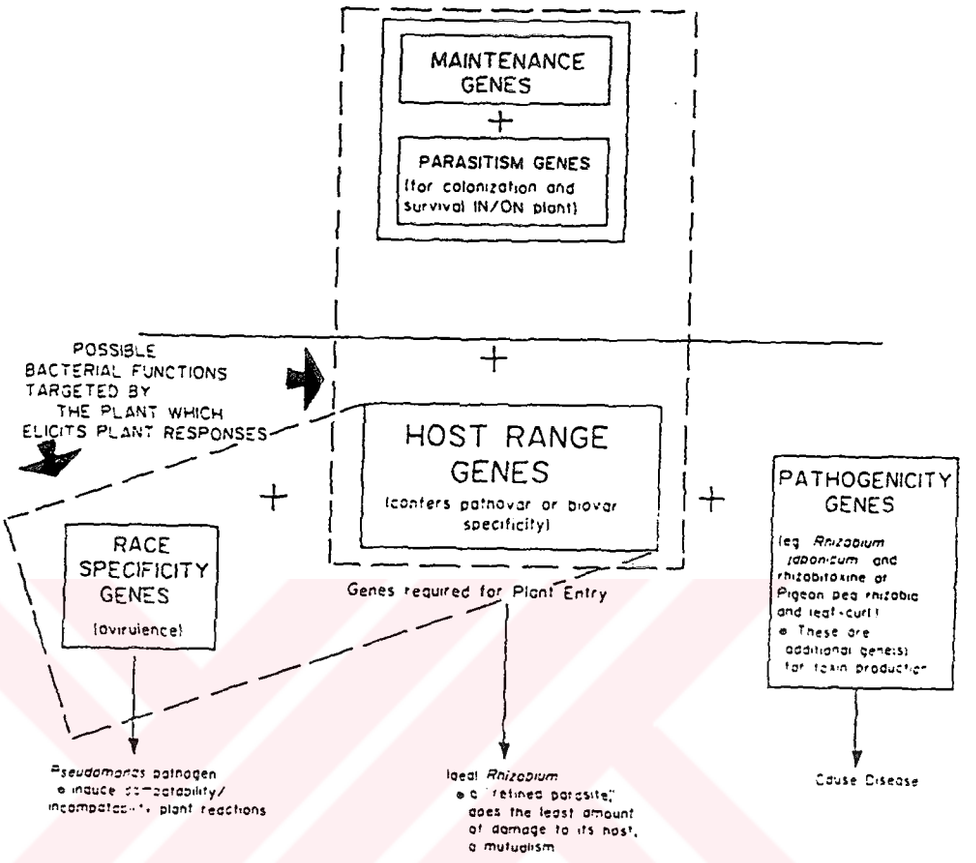


Figure 1.8. Diagrammatic representation showing microbial genes involved in or determining the outcome of bacterial interactions with plants (Djordjevic *et al.*, 1987).

Rhizobium nodulation genes (*nod*) have been defined by sequencing, transposon mutagenesis, and, in some cases, by protein analysis (Michiels and Vanderleyden, 1993; Miksch *et al.*, 1994). Nodulation genes are defined by their effect on the bacterial ability to cause prompt nodulation on the correct host, but they differ in the degree of severity of mutant phenotype. Mutants such as *nod*A, *nod*B or *nod*C are completely *Nod*$^-$ (no nodules formed); these genes are required

for bacteria to initiate cell division (Dudley *et al.*, 1987) and for deformation of root hairs (Djordjevic *et al.*, 1985; Kondorosi *et al.*, 1984; Rossen *et al.*, 1984; Bender *et al.*, 1987). The *nod*ABC genes appear to be functionally interchangeable among all rhizobia (Kondorosi *et al.*, 1984) bacteria carrying mutations in other genes, such as *nod*FE, *nod*G, *nod*H and *nod*LMN, elicit abnormal root hair reactions on their usual hosts and sometimes elicit root hair deformation and even curling on hosts they normally ignore (Djordjevic *et al.*, 1985). These genes are not conserved, since alleles from different rhizobia cannot substitute for each other on different host plants (Djordjevic *et al.*, 1985; Kondorosi *et al.*, 1984). Based on this, *nod* genes are tentatively grouped as "common" and "host specificic" nodulation genes (Horvath *et al.*, 1987).

To discover the genes involved in nodule formation, several research groups have employed transposon mutagenesis in *R. meliloti* (Triplett, 1990; Ulrich and Pühler, 1994). Initial studies, using *R. leguminosarum* bv. *trifolii* strain ANU843, identified genes necessary for root nodulation were located on a large indigenous plasmid species and clustered on about a 14 Kb Hind III fragment of DNA (Djordjevic *et al.*, 1985; Schofield *et al.*, 1984; Innes *et al.*, 1985; Innes *et al.*, 1988). In other rhizobia, the nodulation genes were not as tightly clustered or arranged in the same way as in *R. leguminasarum* bv. *trifolii* (Figure 1.9).

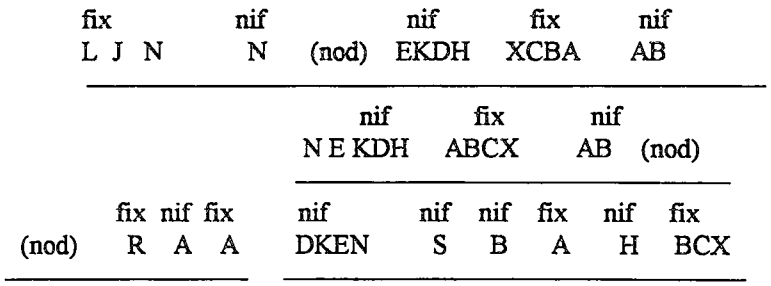


Figure 1.9. Maps showing *nif* and *fix* genes in three organisms with different linkage relations. (Top) *R. meliloti.* (Middle) *R. leguminosarum* biovar *trifolii* and *leguminosarum* (composite). (Bottom) *B. japonicum* (Long, 1989).

The mutation of four contiguous and highly homologous genes designated as *nod*ABC in *R. trifolii* and *R. leguminosarum* resulted in mutants that are unable to curl the root hairs of their respective hosts (*Hac*$^-$ phenotype) (Djordjevic *et al.*, 1985). These mutants have phenotype similar to that of strains completely that the *nod*DABC genes are essential for establishing a parasitic association, as suggested by Long (1984). DNA sequence analysis showed that the *nod*D gene is transcribed divergently to the *nod*ABC operon (Egelhoff *et al.*, 1985). In *R. meliloti nod*ABC mutants were also *Hac*$^-$ (Egelhoff *et al.*, 1985); but the *nod*D gene is reiterated on the *Sym* plasmid. The *nod*D mutants retained the ability to infect, but the nodulation response less efficient and delayed on certain plants. Interspecies complementation studies demonstrated that the *nod*DABC genes were functionally interchangeable between species (Kondorosi *et al.*, 1984) and thus they were called the common *nod* genes.

Constitutive expression of the *nod*D gene has been shown in several species, using *E. coli lac* gene fusion derivatives (Innes *et al.*, 1985; Rossen *et al.*, 1985). The expression of *nod*ABC genes is repressed in normal culture media. For the induction of *nod*ABC genes, both the *nod*D gene product and plant-secreted

phenolic compounds have been required (Figure 1.10) (Innes *et al.*, 1985; Rossen *et al.*, 1985). In several plant-*Rhizobium* systems the inducing molecule have been purified from plant exudates by using ether extraction and reverse phase HPLC chromatography. These compounds, identified as flavonoids, were three ring-aromatic compounds derived from phenylpropanoid metabolism (Figure 1.11). They have been isolated from white clover, lucerne and peas. In most cases, the stimulatory compounds were hydroxylated flavones or flavanones (e.g. 7,4-dihydroxyflavone, clovers; luteolin, alfalfa; apigenin-7-0-glycoside, peas) (Hubac *et al.*, 1993; Milagros *et al.*, 1993). The stimulatory hydroxyflavones were released from the zone of emerging root hairs, and the bacterial *nod* genes were expressed within minutes. That different compounds act as inducers or inhibitors in various legume-*Rhizobium* systems has been substantiated by studies using a variety of purified natural or synthetic compounds (Firmin *et al.*, 1986). Furthermore, the distinguishable responses of various *Rhizobium* spp. to particular compounds have been genetically linked to the particular *nod*D allele. In several cases, it has been shown that different *nod*D genes confer distinctive patterns of *nod* gene response to a variety of pure flavonoids or plants exudates (Bassam *et al.*, 1988; Krischman and Pueppke, 1993). The broadness or specificity of *Rhizobium* response to inducers correlates with their *nod*D alleles (Schiaman, 1992; Sousa *et al.*, 1993).

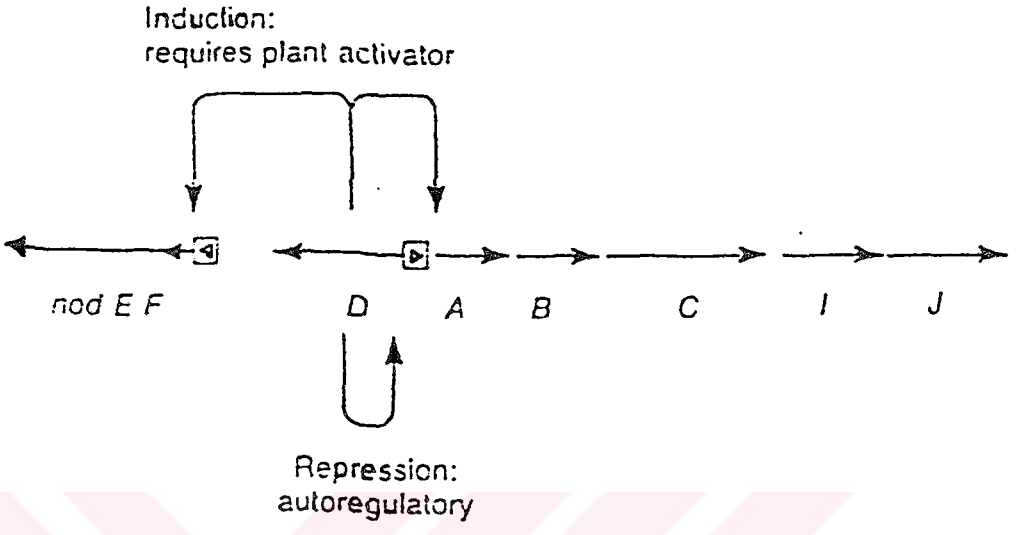


Figure 1.10. Representation of the *nod* genes of *R.leguminosarum* and their regulation (Johnston, 1990).

Genes for nitrogen fixation in *Rhizobium* are generally divided into two groups; those with homologous in free-living nitrogen fixing systems, such as *Klebsiella*, are referred to as *nif* genes; those that are shown to be required for symbiotic nitrogen fixation and whose function is not known to be analogous to a free-living function are referred to as *fix* genes. Both *nif* and *fix* gene mutants are able to cause nodule development, but the nodules do not fix nitrogen (*Nod*$^+$,*Fix*-).

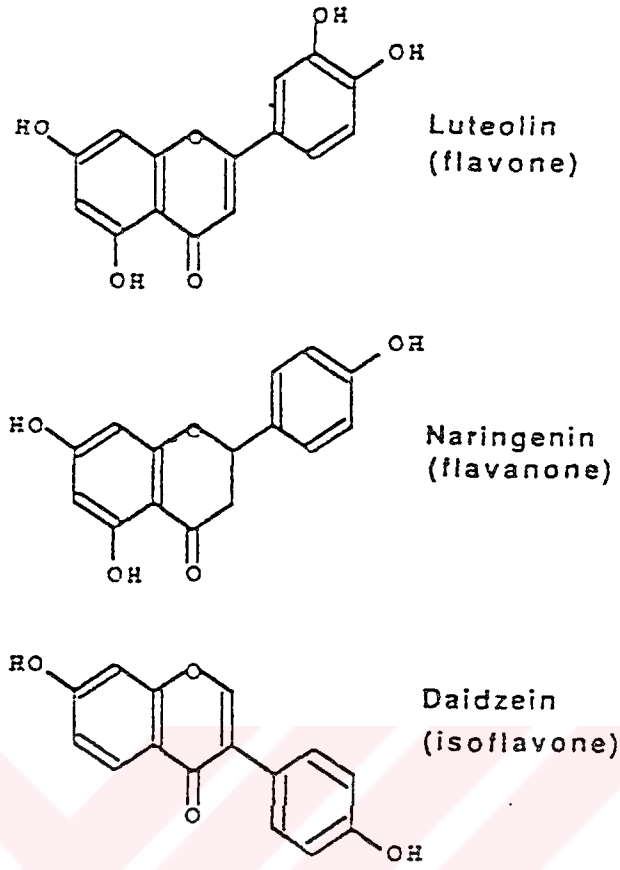


Figure 1.11. Three compounds active as *nod* gene inducers in different systems (Long, 1989).

The development of new genetic techniques and the increased understanding of rhizobia-plant interaction have stimulated an enhanced interest in genetic improvement of rhizobia and/or plant partners. The possible approaches are listed below (Ligon, 1990; Pretorius-Guth *et al.*, 1990; Johnston, 1990).

1. The most promising avenues for increasing symbiotic nitrogen fixation are those that focus on the bacterial partner which is more easily manipulated. One such approach is to increase the number of nitrogen fixation genes with a concominant increase in the number of active nitrogenase molecules. Since the rate-limiting step in nitrogen fixation is the cycle of binding reduced nitrogenase reductase, the *nif* H gene product, to dinitrogenase followed by a one electron transfer, it has been proposed that increased copies of the *nif* H gene would result in increasing the nitrogen fixation activity.

2. Symbiotic nitrogen fixation is limited by the amount of plant-derived photosynthate available to bacteroids. It has therefore be suggested that increasing the ability of endosymbiont to utilize photosynthate in the nodule may lead to increased nitrogen fixation rates.

3. It may also be possible to attain increased symbiotic nitrogen fixation by introducing a more active nitrogenase from another diazotroph. If two *Rhizobium* strains are available, one that fixes a great deal of nitrogen, but is not competitive for nodulation and another for which the reverse is true, transferring the DNA from the first strain to the other might create a hybrid that is both competitive and an efficient nitrogen fixer.

4. It is possible to broaden the host range of an effective strain by transferring *Sym* plasmid from another strain with a different host specificity.

5. One often mentioned and attractive target for future efforts in this field is the creation of plants capable of fixing nitrogen independent of a symbiotic relationship. One serious obstacle to this approach is the necessity to provide protection of oxygen-labile nitrogenase in an aerobic organism.

Conclusively, the genetic manipulation of the symbiotic systems will lead to cheaper and more efficient means of producing high quality plant protein.

Present study aims at an evaluation of symbiotic effectiveness of various strains of *Rhizobium cicer*. Nodulation and nitrogen fixation potential of the strains were assessed and compared. The competitiveness of rhizobia was determined through the experiments which involved the use of mixed inocula. The strains were further characterized in terms of their antibiotic susceptibility patterns and plasmid profiles and the impact of some factors such as pH, inoculum size and minerals on the outcome of symbiosis was also examined.

CHAPTER II

MATERIALS AND METHODS

2.1 Bacterial Strains

A list of bacterial strains used in this study and their sources are shown in Table 2.1.

2.2 Chemicals

The chemicals used and their suppliers are listed in Appendix A.

2.3 Culture Media

Composition and preparation of culture media are given in Appendix B.

2.4 Maintenance of Bacterial Strains

The strains were streaked on Yeast Extract Mannitol Agar (YEM).They were stored at 4°C and subcultured monthly. Cultures were also maintained at -20 °C in YEM broth-15% glycerol.

Table 2.1 List of Bacterial Strains

Strain Designation	Synonyms/Description	Source
R. cicer		
3379	Nitragin 27A8	Prof. Dr. V. Gürgün, Faculty of Agriculture, Ankara University
1aa2	19SA1Ca2	"
11-a	20TKCa1	"
3233	3HOa6, Nitragin 27A12	"
3371	Ca36, Nitragin 27A17	"
TAL 620	IHP3889; CB1189	"
TAL 385	USDA 340 a1; CB1189	"
Y-29	06 Gu Ca1	"
3378	Nitragin; 27A7	"
R. leguminosarum		
2517	1027(ade,phe,nif) pVW5J1(=pRL4J1::Tn5)	Dr. N. J. Brewin, John Innes Institute Colney Lane, Norwich U.K
T3	1062(ura,trp,str) pJB5J1(pRL1J1::Tn5)	"
A. tumefaciens	LB4440	Dr. H. A. Öktem, Biology Dept., M.E.T.U., Ankara
R. phaseoli	193	U.S.D.A (U.S.A)

2.5 Buffers

The buffers used in this study and their composition are presented in Appendix C.

2.6 Growth Measurement

Bacterial growth was measured as absorbance at 600 nm using a Bausch-Lomb Spectronic 20 spectrophotometer. The cultures were appropriately diluted with fresh media before measurements and growth was expressed as A_{600} multiplied by the dilution factor.

Viable counts were made by serially diluting the 16 h cultures and plating 0.2 ml portions of the dilutions onto YEM agar plates. A calibration curve was constructed by plating the absorbencies against viable cell number (colony forming units per ml) (Figure 2.1)

2.7 Batch Culture and Construction of Growth Curve

A seed culture was started by transferring single colonies from an overnight YEM agar culture into 20 ml of YEM broth in a 100 ml Erlenmayer flask. The culture was grown for 16 h at 28°C on a reciprocal shaker (50 strokes per min). Samples were taken from the culture at 4 h intervals and optical densities were measured.

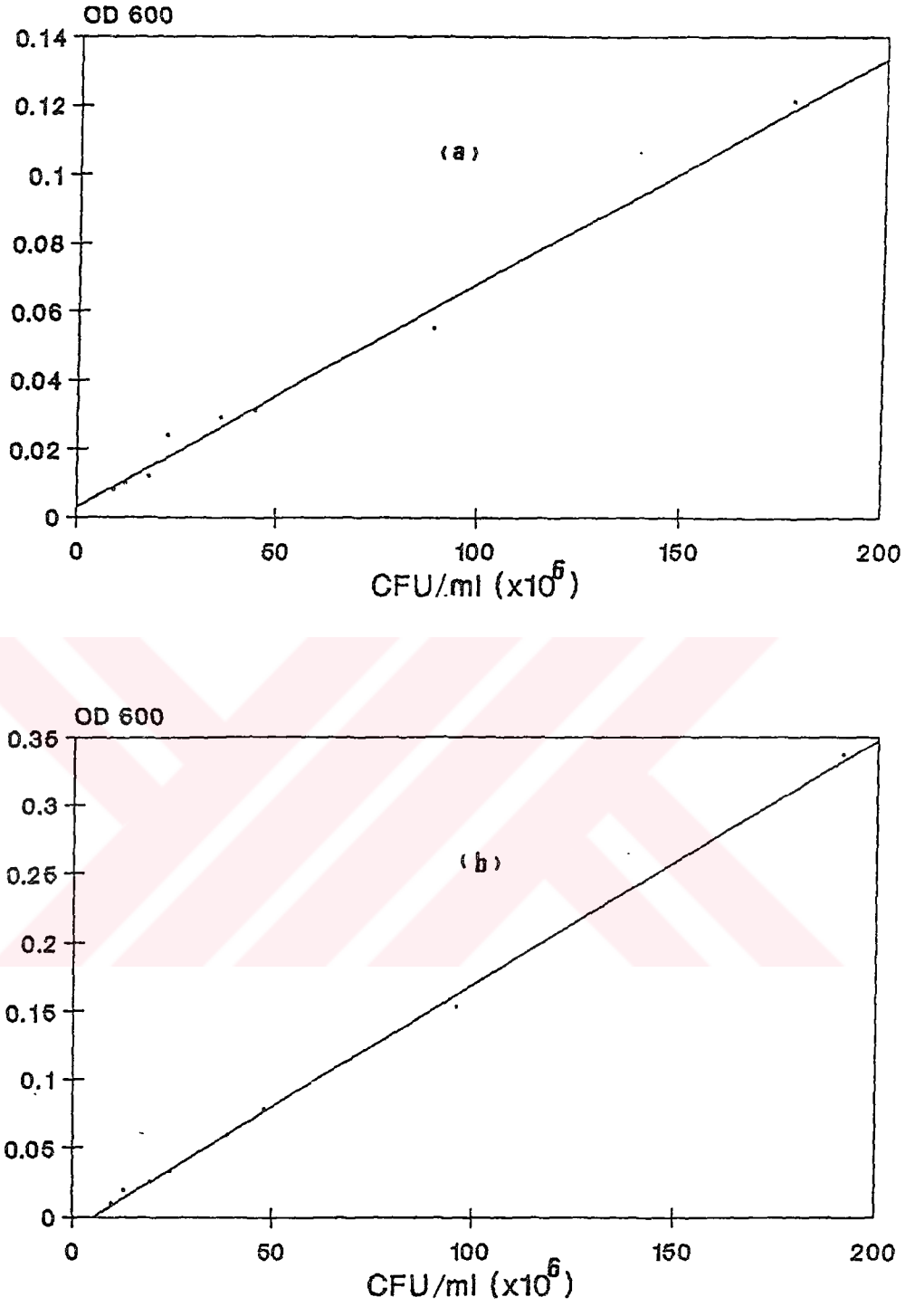


Figure 2.1. Absorbance versus viable cell number calibration curves for *R.cicer* strains 385 (a) and 620 (b).

2.8 Plant Seeds

A batch of chickpea (*Cicer arietinum* L.) seeds were kindly supplied by Dr. Kamuran Ayhan, Faculty of Agriculture, Ankara University. The seeds have been designated ESER 87 by the supplier.

2.9 Surface Sterilization of Seeds

Before pursuing to surface sterilization, healthy seeds were selected and cleaned. The seeds were then placed in a sterile Erlenmeyer flask. The seeds were rinsed in 95 % alcohol for 10 seconds to remove waxy material and then the alcohol was drained off. Hydrogen peroxide solutions in sufficient volumes to completely immerse the seeds were added. The contents were swirled gently to bring the seeds and sterilant into proper contact. After 3-5 minutes, the sterilant was drained off. The seeds were rinsed with at least six changes of sterile water. After the sixth rinse, the flask was flushed with sufficient water to submerge the seeds and left in the refrigerator for 4 h for the seeds to imbibe. Finally, the seeds were rinsed with two or more changes of water.

2.10. Seed Germination

Chickpea seeds were placed on 0.75 % (w/v) agar in petri dishes. They were spread out on the agar to avoid over-crowding. Plates were incubated at 25-30°C .

2.11. Planting of Seeds and Infection

2.11.1. Preparation of Leonard Jars

The modified Leonard Bottle-Jar assembly consisted of a 700 ml capacity bottle with the lower portion cut off (Figure 2.2). This was inverted into a heavy glass jar (reservoir) with a capacity of a liter or greater in such a way that the mouth

of the bottle was about a 2-3 cm above the base of the reservoir. The growth medium in the bottle was irrigated by a centrally positioned cotton wick which was run through the length of the bottle and extended out of the mouth of the bottle into the reservoir containing the nutrient solution. Cotton wick was chosen for its ability to conduct water and for being compatible with plants.

About 45-50 cm of wick material was placed in the bottle with about 10 cm extending out of the mouth. A small amount of absorbent cotton stuffed into the neck of the bottle was aided in securing the wick in position and at the same time prevent the settling of the growth medium into the reservoir. The cotton wick was boiled in water and squeezed dry prior to use. While holding the wick in a central position, the bottle was filled with well-washed river sand. The sand was packed to minimize air spaces. The bottle was placed in position in the reservoir. The sand in the bottle was moistened by pouring 150-200 ml of the nitrogen-free nutrient solution (Jensen Medium) (Appendix B). The medium was allowed to saturate with the nutrient solution and the excess to drain into the reservoir. The reservoir was filled with 800 ml of the nutrient solution . The bottle jar assembly was wrapped with moisture proof paper and the wrappings were secured with rubber bands. The open end of the bottle was capped with aluminum foil. The complete assembly was sterilized by autoclaving, for 1.5-2.0 h at 121°C and 15 psi.

2.11.2. Preparation of Inoculants

R. cicer strains were grown in 100 ml of YEM broth in 250 ml flasks. The cultures were shaken for about 16 h to a cell density of 10^9 cells/ml.

2.11.3. Planting and Inoculation of Seeds in Leonard Jars

Surface sterilized and pregerminated chickpea seeds were selected by considering their uniform size and high viability. The well-germinated seeds of similar size were selected. Three seeds were transferred aseptically to each Leonard Jar. The Leonard Jars were then inoculated with bacterial suspension (1ml/seed).In all experiments conducted throughout this study the jars were run in triplicates. Inoculated Leonard Jars were incubated in a controlled-environmental chamber (Heraeus Votsch) at 25°/24°C (day/night) with a humidity of 70 % for 30 days. After 5-7 days old, the Leonard Jars were examined. The jars with poor plants were discarded. A plus-nitrogen control (50g natural fertilizer) inoculum and an uninoculated control were also included.

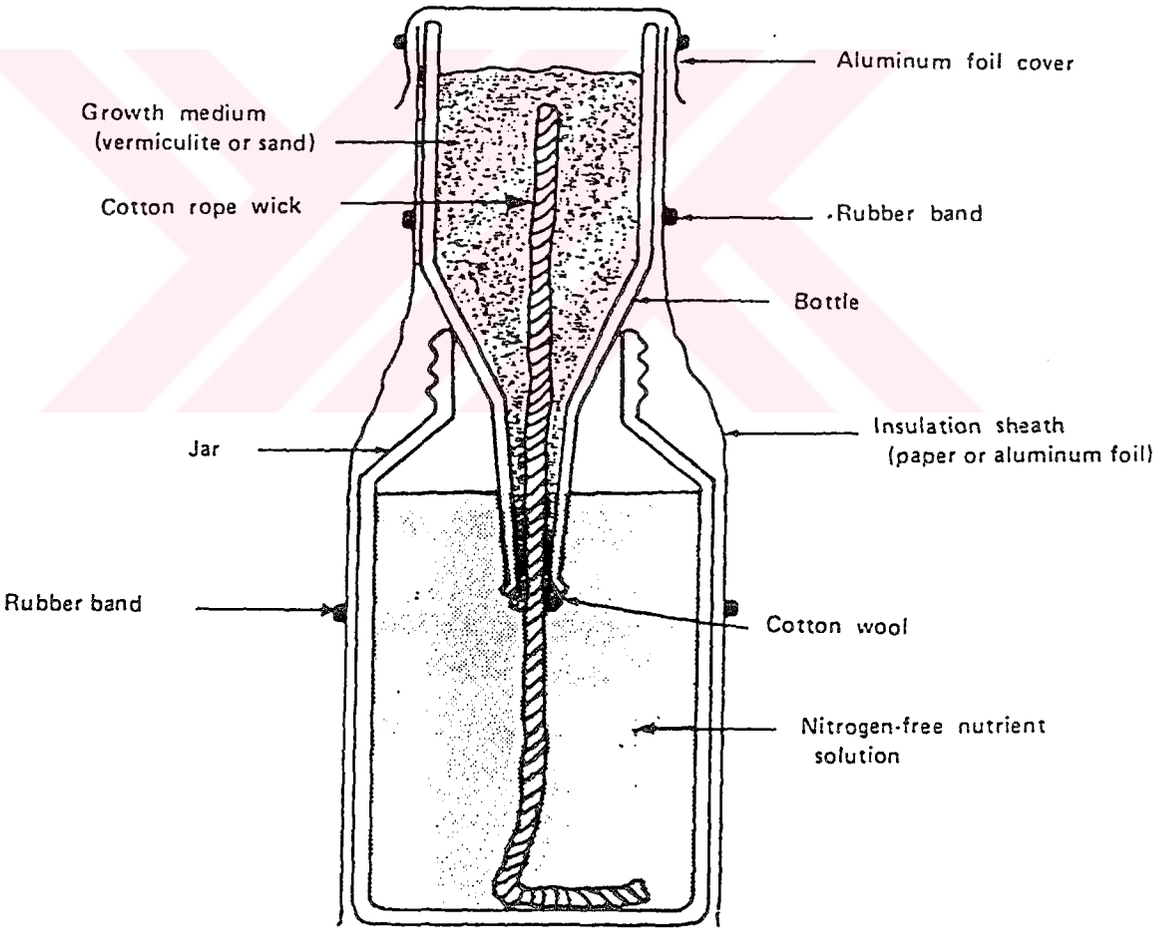


Figure 2.2. The Leonard Jar diagram.

2.12. Harvesting of Plants

Plants were harvested after a growth period of 30 days. To minimize errors during harvest, the stems were cut at the point of cotyledon attachment. Plant shoots were cut and placed in separate petri dishes. The shoots were dried to a constant weight at 70°C for 24 h. Nodules were detached from the roots, counted and weighed. They were then placed into petri dishes, dried and weighed.

2.13. Determination of Nitrogen Content by Macro Kjeldahl Method

The nitrogen content of plants was determined by Macro-Kjeldahl method after Bremner (1965).

A well-ground sample containing ca. 10 mg of plant shoot was placed in a Macro-Kjeldahl flask (Figure 2.3). Then 20 ml of water was added and after swirling the flask for a few minutes the mixture was allowed to stand for a further 30 min 10 g of K_2SO_4, 1 g of $CuSO_4$ H_2O, 0.1 g of Se and 30 ml of concentrated H_2SO_4 were then added and the flask was heated cautiously on the digestion stand. After the water had evaporated and frothing had ceased, the heat was increased until the digest cleared, and thereafter the mixture was boiled gently for 5 hours. The heating was regulated during this boiling so that the H_2SO_4 condensed about one-third of the way up the neck of the flask.

After completion of digestion, the flask was allowed to cool and about 100 ml water was added slowly, and with shaking. Then the flask was cooled under a cold water tap, and the contents were transferred to a 1-litre conical (or 800-ml Kjeldahl) flask for distillation.

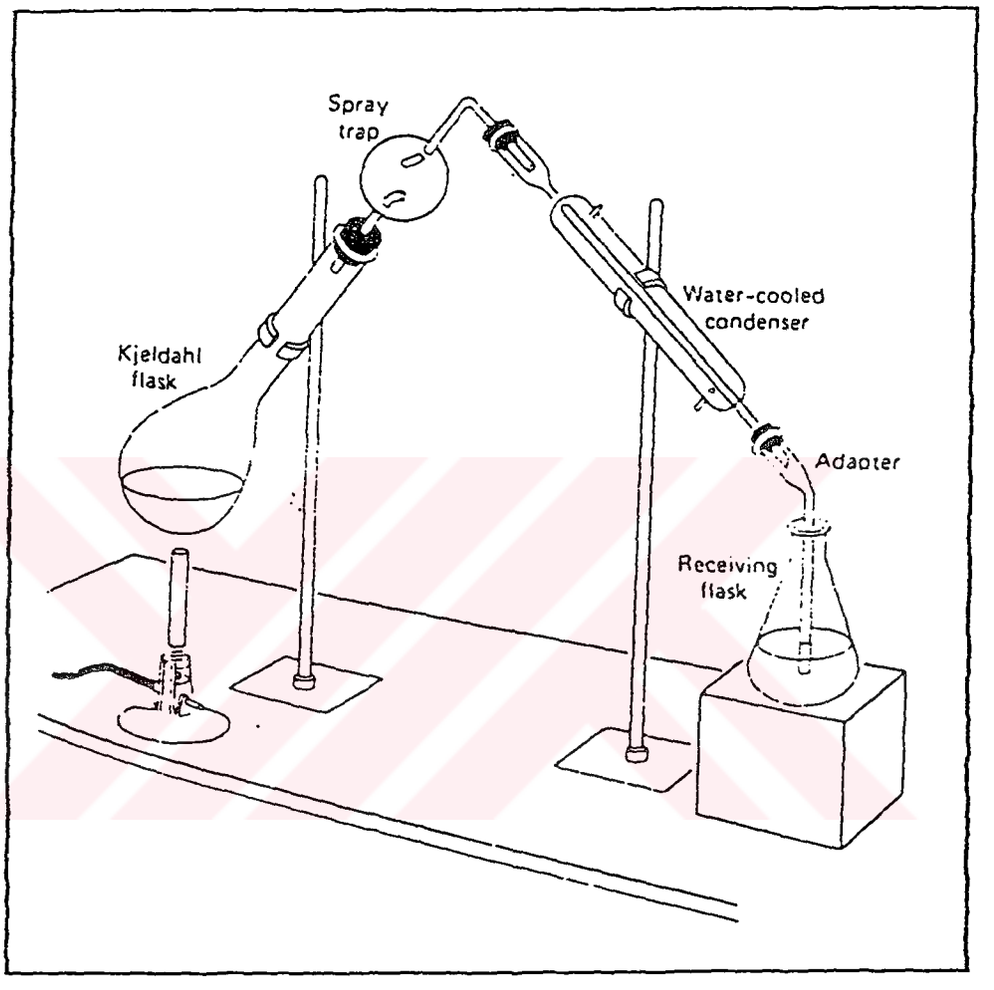


Figure 2.3. The Kjeldahl Apparatus.

To determine the ammonium-nitrogen liberated by digestion, a 500 ml Erlenmeyer flask containing 50 ml of H_3BO_3-indicator solution was placed under the condenser of the distillation apparatus so that the end of the condenser was below the surface of the H_3BO_3. Then the distillation flask was held at a 45° angle and a teaspoonful of pumice was added. About 150 ml of 10 N NaOH was poured down the neck of the distillation flask so that the alkali reached the bottom of the flask without mixing appreciably with the digest. The flask was attached as quickly as possible to the distillation apparatus after mixing the contents thoroughly by swirling and distillation was commenced immediately. The heating was regulated to prevent suckback of H_3BO_3 and to minimize frothing or bumping during distillation and the flow of cold water through the condenser was checked to keep the temperature of the distillate at 35°C. When about 150 ml of distillate had been collected, the receiver flask was lowered so that the end of the condenser was above the surface of the distillate and after rinsing the end of the condenser with water, the flask was removed and distillation was stopped. The ammonium-nitrogen content in the distillate was determined by titration with 0.05 N H_2SO_4 following the color change of the indicator from green to pink, and 1ml of 0.05N H_2SO_4 was considered as the equivalent of 0.7 mg ammonium-nitrogen.

2.14. Determination of Total Protein Content

Samples were prepared for protein determination according to the method of Gegenhermer (1990). 0.05 g of the plant dry shoot was homogenized by freezing in liquid nitrogen and grinded to a dry powder in a mortar. 10 ml of 10 % TCA was added to the sample which was then mixed and transferred to a Corex tube. After incubation for 1 h at room temperature, samples were centrifuged at 10000 rpm for 10-15 min in a Sorvall RC-F centrifuge. Pellet was taken and 10 ml of pure acetone was added to remove chlorophyll. The samples were recentrifuged at 10000 rpm for

10 min. The acetone washing was repeated four times. After drying under vacuum for 10 min 5 ml distilled water was added to 0.1 g of powdered sample, to obtain a homogenous mixture.

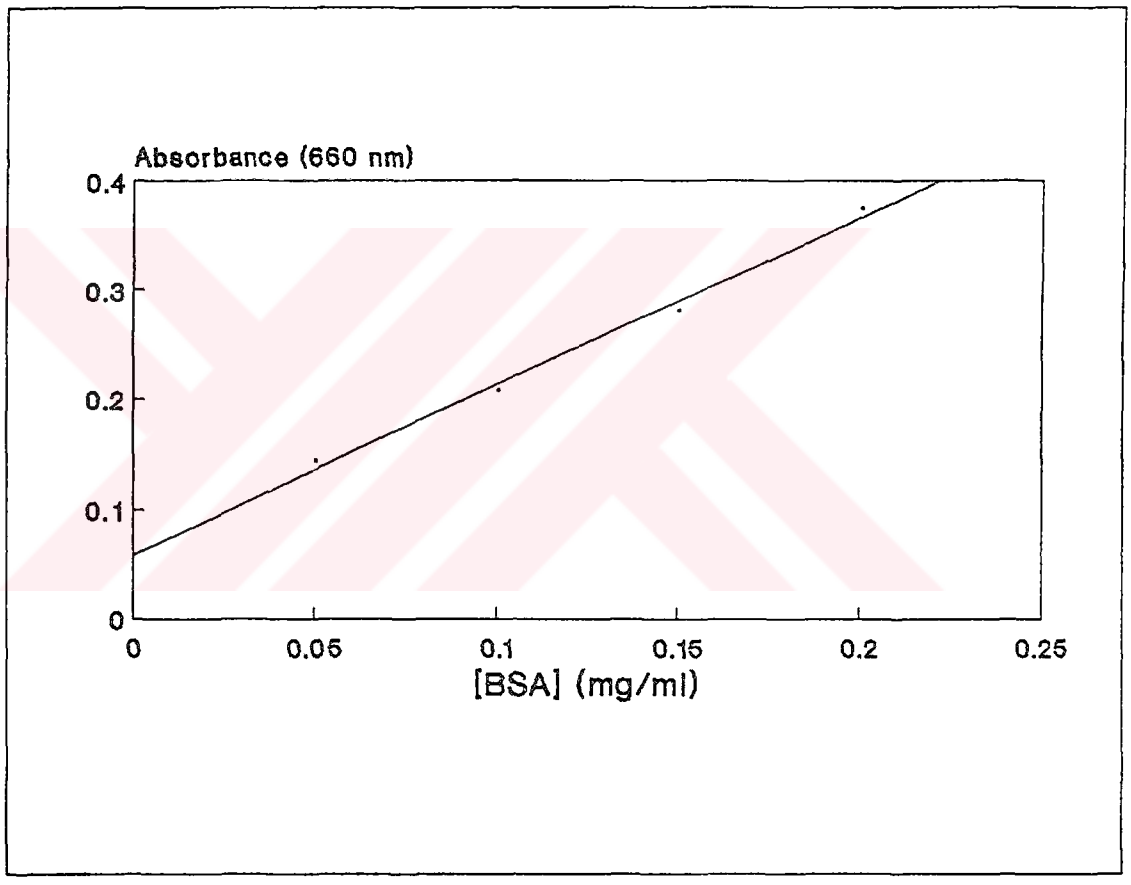


Figure 2.4. Calibration curve for total protein determination.

The quantitative determination of plant protein was performed by using Lowry method (Lowry, 1951). Copper sulfate, sodium potassium tartarate and sodium carbonate (each at 2 % concentration w/v) were mixed in 1:1:100 ratio, just before use. 5 ml of this reagent was added to 1 ml of the sample containing 2 mg of extract. The mixture was allowed to stand for 10 min at room temperature. Folin reagent was diluted with water (1:1) and added in 0.5 ml volume to the mixture. After 30 min at room temperature, absorbance at 660 nm was measured in a Bausch-Lomb Spectronic 20 spectrophotometer. Bovine Serum Albumin (BSA) was used as a standard (Figure 2.4).

2.15. Evaluation of Results

In order to show the relationship between shoot dry weight, nodule dry weight and nodule number per seed, correlation coefficients (at 0.5% and 0.1% confidence levels) were computed for each tested parameter. The significance of each treatment was also tested as described by Somasegaran and Hoben (1985).

2.16. Mixed Infection

Chickpea seeds were also inoculated with a mixture of different strains. The mixed inocula comprised the effective strains selected in previous experiments. The density of cultures was determined spectrophotometrically. 1 ml of mixed inoculum was adjusted to contain 10^8 cells of each strain and used to inoculate each seed.

2.17. Determination of Antibiotic Resistance Patterns

Stock solutions of antibiotics were prepared from the material of known potency (Sigma grade). The antibiotics were then incorporated into YEM agar at different concentrations. YEM agar plates containing antibiotics were inoculated

with 0.1 ml of overnight YEM broth cultures of *Rhizobium* strains. The plates were incubated at 28°C for 24-48 h.

2.18. Determination of Competitiveness of Strains

The plants were double-infected and nodulation competitiveness of strains was determined by comparing the number of nodules occupied by each strain.

At the time of plant harvesting, roots containing nodules were surface sterilized by shaking for 45 seconds with sodium hypochloride (5 % w/v). Each root was then rinsed twice in sterile water, and placed in a sterile petri plate. Individual nodules were stabbed with sterile toothpicks. The contents of each toothpick were spotted on grid-marked TY agar plates which will be used as the master plates. The plates were incubated for 24 h at 28°C and the developed colonies were transferred onto TY agar plates containing appropriate antibiotics.

To determine rhizosphere competitiveness, each nodulated root was placed in a sterile test tube. Then 10 ml of saline solution (0.85 %, w/v) was added into each tube and washed by gentle vortexing. The solution was serially diluted and 0.1 ml aliquots were plated on TY agar containing the appropriate antibiotics. The colonies on each plate were counted after an incubation period of 24 h.

2.19. Bacteriocin Production

The strains tested for bacteriocin production were grown as single colonies on YEM-agar plates for 48 h at 28°C. They were then screened for bacteriocin production by Hirsch (1978).Single colonies were transferred to fresh YEM agar plates by using sterile toothpicks. The plates were incubated for 2 to 4 days until a patch of growth 3 mm in diameter was visible at each plate. Regrowth was

prevented by surface-sterilization. For this purpose, plates were inverted over glass petri dishes containing 5 ml chloroform for 45 min. and aired for 30 min. Each plate was overlaid with 2.5 ml of soft YEM agar containing (0.7%, w/v) the indicator strain. The indicator strain was grown to form a confluent lawn and the plates were checked for the presence of inhibition zones.

2.20. Plasmid DNA Isolation

Casse and Boucher (1979)'s procedure was used for the isolation of high molecular weight plasmid DNA.

To avoid the formation of excess polysaccharides, bacteria were grown in 50 ml of TY medium. They were harvested before the end of the exponential phase. Then NaCl was added at a final concentration of 1 M, and the culture was shaken vigorously for 30 min. Bacteria were washed twice with TE buffer (pH 8.0) and the pellet obtained was weighed and suspended in TE buffer, pH 8 (100 mg bacteria to 0.5 ml buffer for better reproducibility). The lysing buffer (TE buffer containing 1 % (w/v) sodium dodecyl sulfate) was adjusted to pH 12.45. To 0.5 ml bacterial suspension in a 50 ml beaker, 9.5 ml of the lysing buffer was added and the mixture was stirred at 100 rev min^{-1} for 90 sec before incubation at 34°C for 20 to 25 min. The pH was then lowered to 8.5 to 8.9 by adding 0.6 ml of 2 M-Tris-HCl buffer (pH 7.0). The lysate was then adjusted to 3 % (w/v) NaCl and after 30 min, 10 ml phenol (previously saturated with 3 % (w/v) NaCl in water) was added. The two phases were mixed by stirring at 300 rev min^{-1} for 10 sec and further stirred for 2 min at 100 rev min^{-1} The mixture was then centrifuged at 5000Xg (6500 rpm) for 10 min and the clear aqueous upper phase was transferred to a sterile Corex tube. Sodium acetate solution was added to obtain a final concentration of 0.3 M and 2 volumes of cold (-20°C) 95 % (v/v) ethanol were added to precipitate the DNA. The tube was

kept at -20°C overnight. The precipitated DNA was recovered by centrifuging at 10000 rpm at -10°C for 20 min. The ethanol was removed from the tube and the DNA pellet was dissolved in 100 µl TES buffer pH 8.0. The tubes were held under vacuum for 5 min to remove residual ethanol. The DNA samples were analyzed immediately by agarose gel electrophoresis. Molecular sizes of plasmids were estimated by consulting to the standard curve (Figure 2.5).

2.21. Agarose Gel Electrophoresis

Electrophoresis was carried out on a horizontal gel apparatus and performed in 0.7 % (w/v) agarose in TBE buffer at 40 V for 1 h and then at 100 V for 5 h. The gel was stained with ethidium bromide solution (1 µg/ml) for 15 min and destained for 10 min in distilled water. The DNA bands were visualized on a short-wave U.V transilluminator.

2.22. Transformation

Transformation of *R. cicer* 620 was performed by the method described by Kiss and Calman (1982). 620 was inoculated into 200 ml TY medium supplemented with 0.3 M sucrose and incubated for 16 h at 28°C to a cell density of $1x10^9$ to $2x10^9$. Cells were collected and suspended in 20 ml of 100 mM $MgCl_2$. After 10 min at 0°C, the suspension was pelleted and suspended in 0.5 ml of $CaCl_2$ (150 mM). After 30 min at 0°C, 100 µl of *R leguminosarum* T3 plasmid DNA (1.0 µg/0.1 ml) was added, and incubation was continued for 60 min. The cells were heat shocked at 40°C for 1.5 min and then incubated for 30 min at 0°C. Bacteria were plated onto selective plates (YEM agar supplemented with 60 µg of kanamycin sulfate/ ml) and incubated.

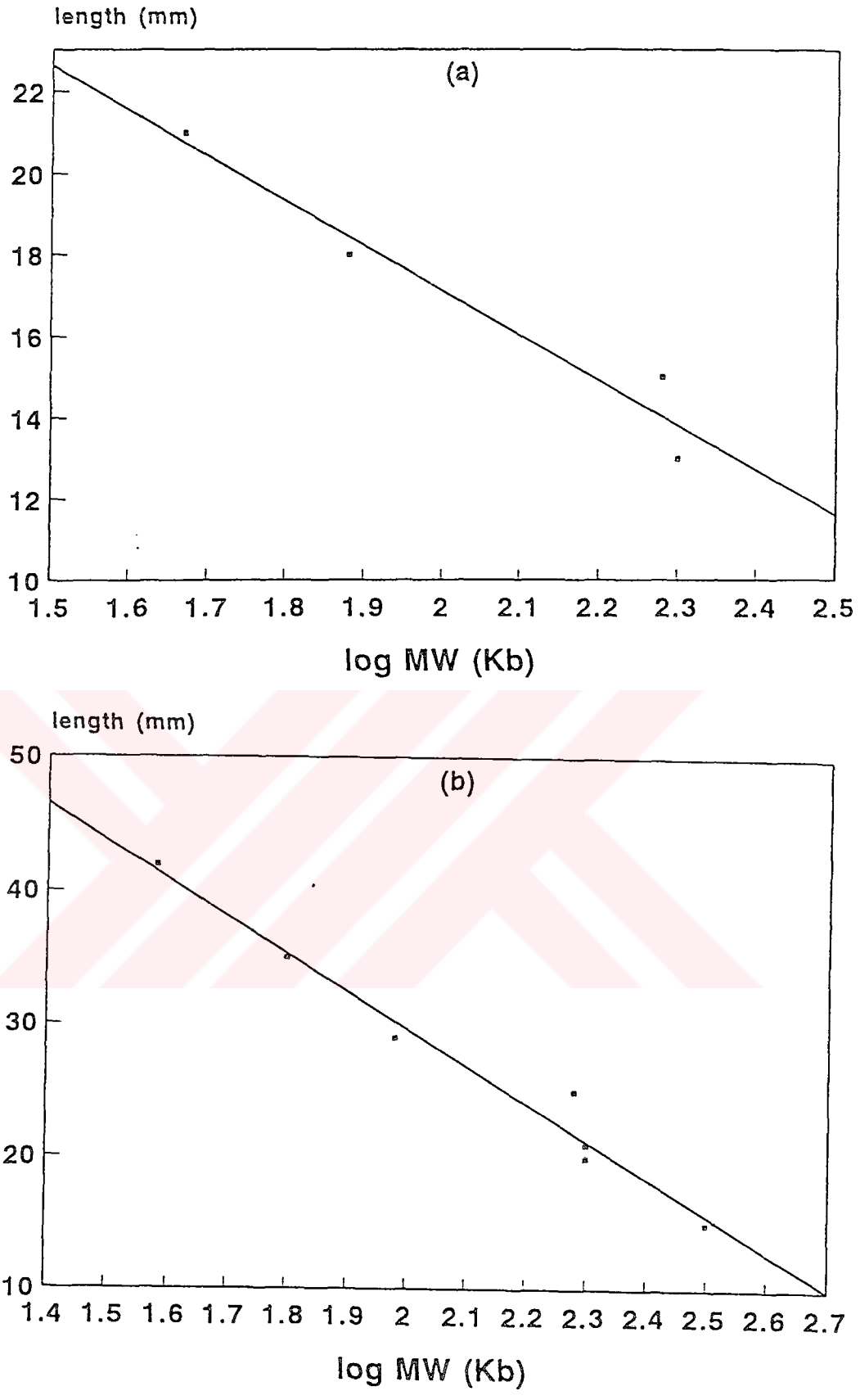


Figure 2.5. Plasmid molecular size estimation standard curve (a) 620 and (b) 3233

2.23. Conjugation

Conjugation was performed by the methods described by O'Connell *et al.* (1987). Donor and recipient strains were grown until late-logarithmic phase and were mixed in 1:1 ratio in a microfuge tube. The mixture was centrifuged at 5000 rpm for 10 min. Supernatant was discarded and the pellet was resuspended in 50 µl TY medium. The suspension was then placed onto the surface of a sterile Sartorious filter paper disc (0.2 µm pore size) which has been placed on a TY agar medium. The plate was incubated for 20 h at 30°C. After the filter was immersed in 5 ml of distilled sterile water in a sterile tube, the dilutions were made and spread onto antibiotic-containing plates for the selection of transconjugants. The plates were then incubated and conjugation frequency was estimated.

2.24. Nodulation in Agar Stabs

A simple technique adopted from Lakshminarayana *et al.* (1988) was used to obtain nodulation in agar stabs. The media used for preparing agar deeps was quarter-strength Jensen medium. The medium was supplemented with 0.8% (w/v) agar and autoclave sterilized. The flask containing the molten medium was immersed in a water bath maintained at 50°C then shaken vigorously by hand so as to generate as many air bubbles as possible. Precisely at this stage the medium was poured into 280x25 mm test tubes to 3/4 volume. All manipulations were carried out under aseptic conditions. An agar tube prepared in this way, contained numerous air bubbles distributed throughout the solidified agar medium. After planting the inoculated pre-germinated seeds in the tubes, they were then wrapped with opaque paper to cover the agar portion and incubated in a greenhouse-chamber.

CHAPTER III

RESULTS

3.1. Morphological Characterization

When the strains of *Rhizobium* were grown on standard yeast mannitol (YEM) agar medium, the colonies were typical in their size, color, shape and texture (Somasegaran and Hoben, 1985). Except for strain 3379, the strains gave domed, smooth-margined, white-opaque to watery- or milky-translucent, gummy and soft colonies. The general pattern of growth in solid media was as shown below (Figure 3.1).

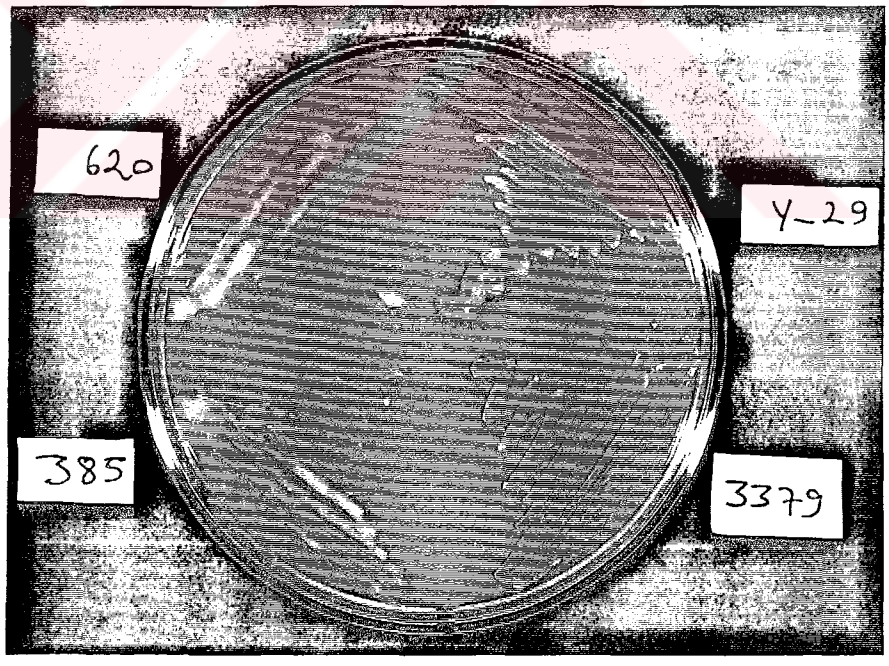


Figure 3.1. Typical growth of *Rhizobium* strains on YEM agar medium.

As stated in the previous chapters, rhizobia do not absorb Congo Red when plates are incubated in the dark. Colonies remain white, opaque or occasionally pink while the contaminant organisms usually absorb the red dye. *R.cicer* 3379 was found to absorb Congo Red. The colonies of other strains remained white (Figure 3.2).

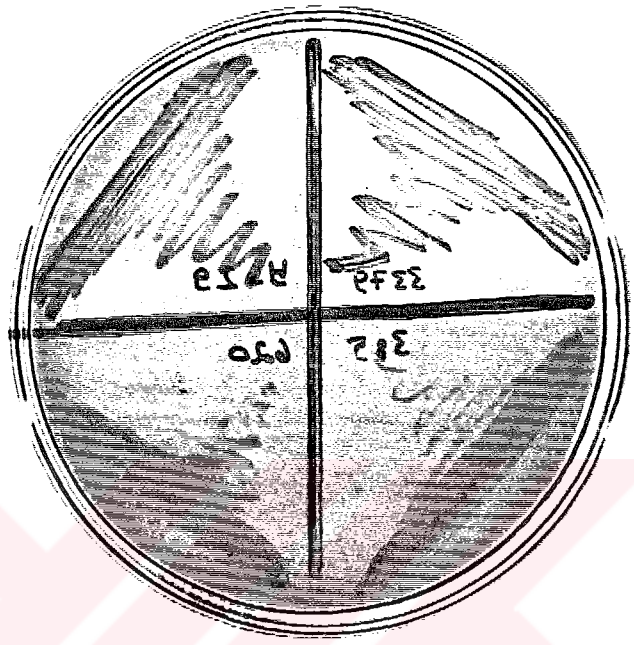
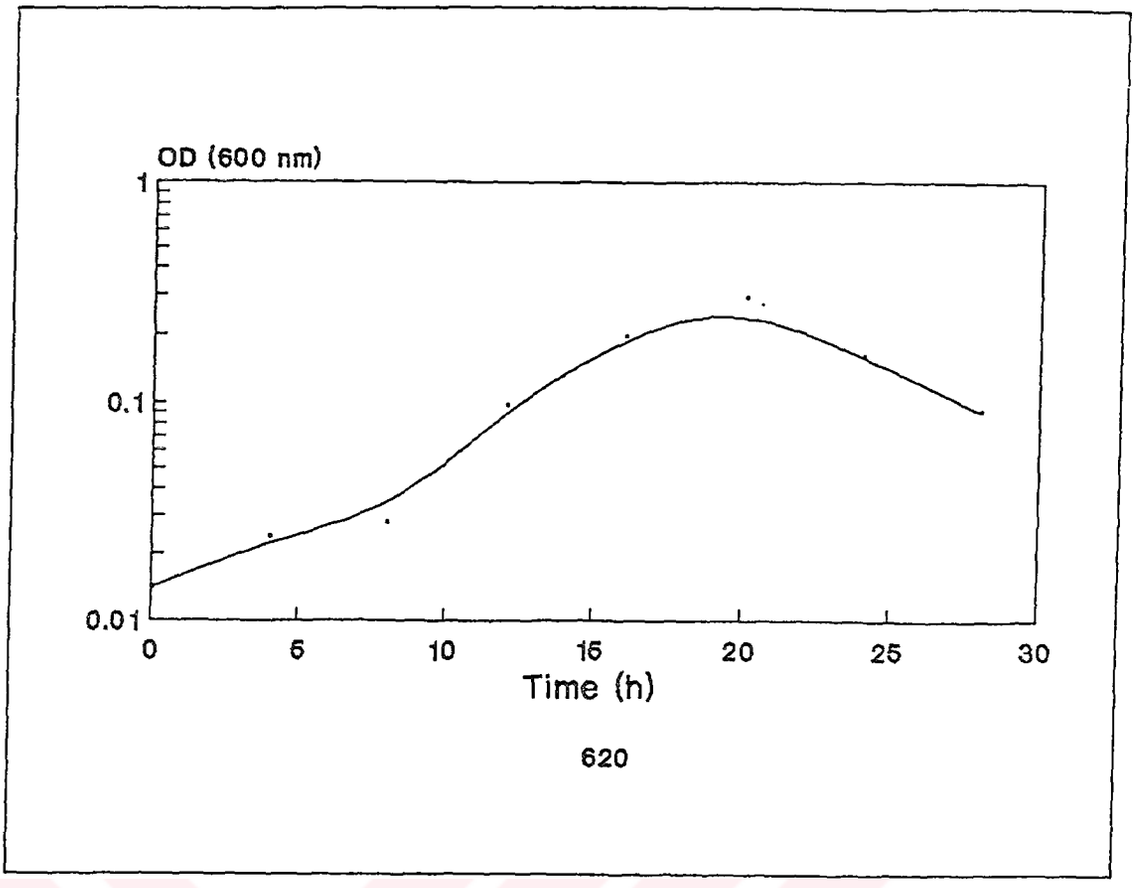


Figure 3.2. Characteristics of *Rhizobium* strains when grown on YEM agar containing Congo Red.

Freshly prepared YEM plates containing bromothymol blue (BTB) had a pH of 6.8 and were green in color. As expected, the strains gave an acid reaction, turning the color of medium to yellow.

3.2. Batch Cultures

The growth curves of strains 620 and 385 were constructed by plotting absorbance at 600 nm against time (Figure 3.3 a and b). Batch culture kinetics of the strains were calculated from the absorbencies and are presented in Table 3.1.



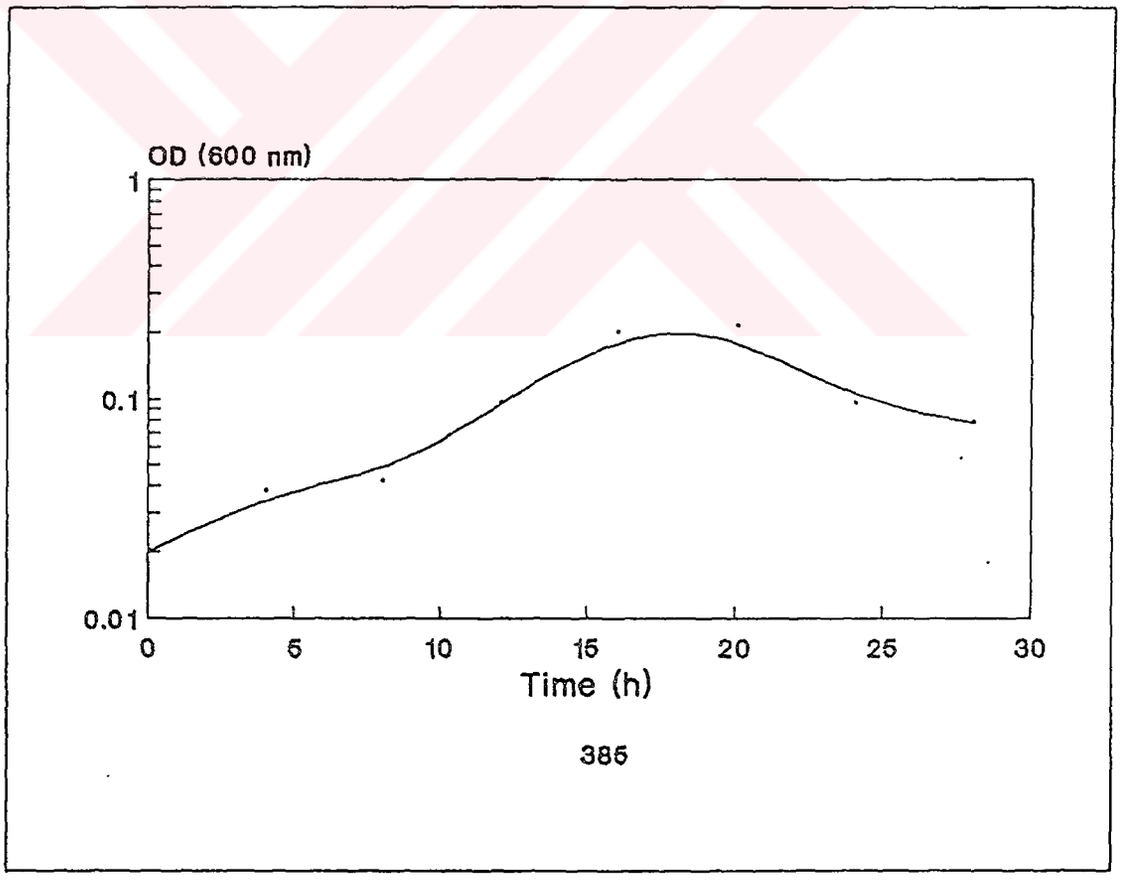


Figure 3.3. Growth curves of *R.cicer* strains 620 and 385

Table 3.1. Specific growth rate and doubling time of strains when grown in YEM broth.

Strain	Specific growth rate (h^{-1})	Doubling time (h)
620	0.165	4.20
385	0.145	4.77

3.3. Antibiotic Resistance Patterns of Strains

Antibiotic resistance patterns of the strains were determined to constitute a basis for further genetical studies which involved investigation of the competitive nature of the strains. The antibiotics, their concentration and resistance patterns are tabulated in Table 3.2. Sensitive strains of *E.coli* and *S.aureus* were used as controls.

Table 3.2. Antibiotic resistance patterns of *R.cicer* strains [a]

Strains Ant. (µg/ml)	Y-29	620	3379	385	1aa2	3378	3371	3233
Str 40	R	R	S	R	S	S	S	S
Str 75	S	S	S	S	S	S	S	S
Spc 100	R	S	S	S	R	S	S	S
Spc 200	R	S	S	S	S	S	S	S
Lin 15	R	R	R	R	R	R	S	R
Lin 25	R	R	R	R	R	S	S	R
Lin 50	R	R	S	R	R	S	S	S
Kan 60	S	S	S	S	S	S	S	S
Chl 30	R	S	S	S	R	S	S	S
Clox 75	R	R	S	R	R	R	R	S
Gen 40	S	S	S	S	S	S	S	S
Nal 25	R	R	R	R	R	R	S	R
Tet 15	R	S	S	S	R	S	R	S
Neo 40	S	S	S	S	S	S	S	S
Ami 75	S	S	S	S	S	S	S	S
Amp 75	R	S	S	S	R	S	S	S

[a] R, resistant; S, sensitive

Sensitivity to most of the aminoglycoside antibiotics (kanamycin, gentamicin and neomycin) was common among the strains. The effective strains were resistant to streptomycin (40μg/ml). Except for 3371, all the strains also displayed nalidixic acid resistance. The strains, on the other hand, markedly varied in terms of their susceptibilities to other antibiotics; lincomycin, chloramphenicol, cloxacillin, tetracycline, ampicilin, and amikacin.

3.4. Bacteriocin Production

Bacteriocin production by *R.cicer* strains was next investigated by determining the cross-sensitivity amongst the strains in our collection. None of the strains gave an inhibition zone when tested against the other strains including some tester strains of *E.coli* (Figure 3.4).

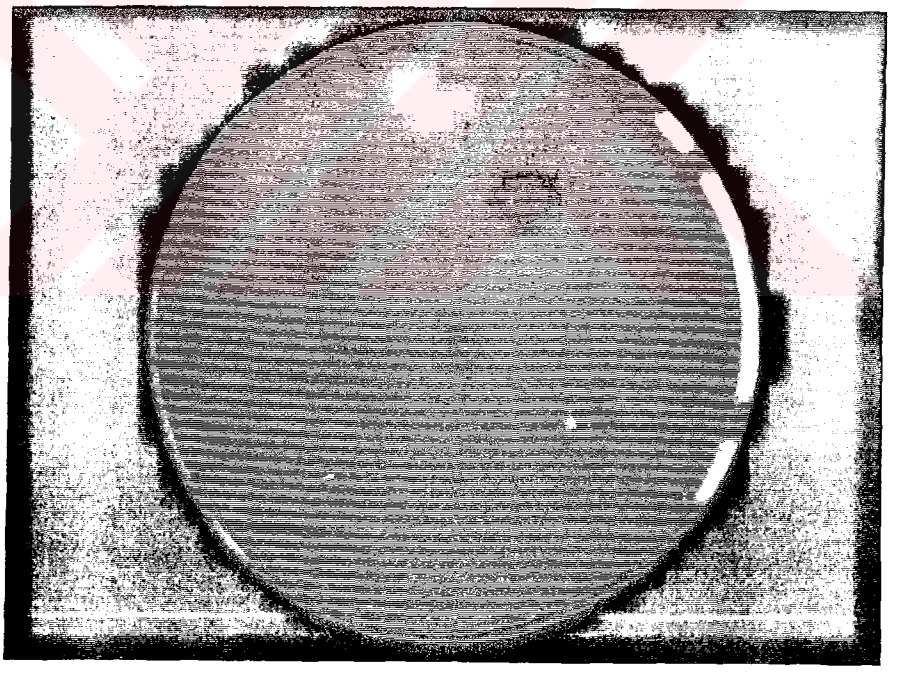


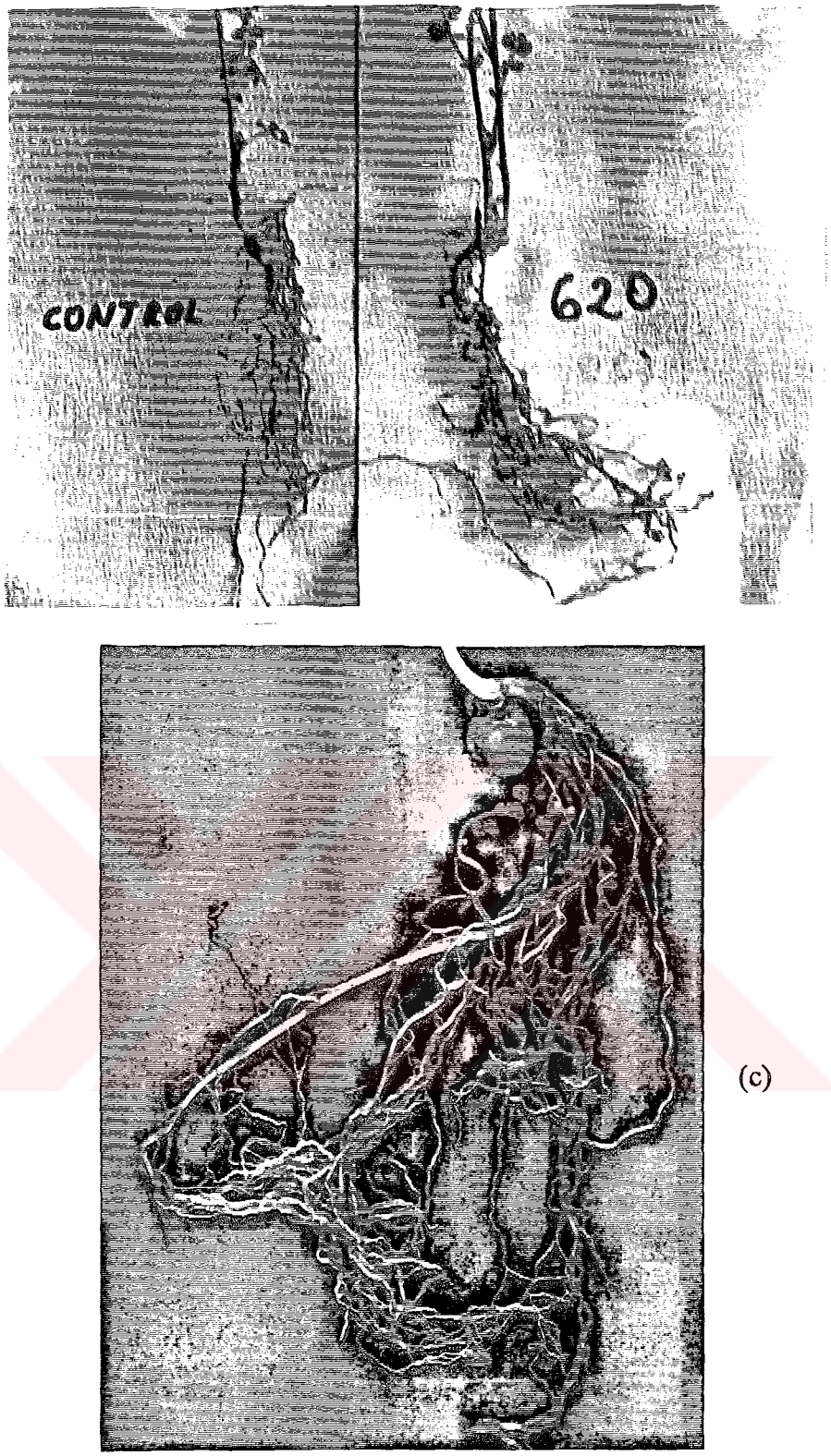
Figure 3.4. Bacteriocin production test.

3.5. Selection of Effective *R.cicer* Strains

Two local isolates (1aa2 and 11-a) and five standard (385, 620, 3233, 3371, 3378) *R.cicer* strains were tested for their ability to nodulate chickpea thus improving the yields of this plant. The assessment of plant productivity was based on shoot dry weight, nodule number and nodule dry weight (Figure 3.5). Strain 385 was found to be the best as it significantly increased shoot dry weight and gave the highest number of nodules. All the criteria of plant productivity were in good correlation in plants infected with this organism (Table 3.3, Figure 3.6). Strain 620 was also fairly effective in nodulating plants. In view of these findings, *R.cicer* 385 and 620 were chosen for further studies.

Table 3.3. Effectiveness of various *R.cicer* strains.

Strain	Nodule number	Shoot dry weight (g)	Nodule dry weight (g)
620	68	0.550	0.14
385	88	2.780	0.20
1aa2	-	0.400	-
11-a	-	0.475	-
3233	-	0.700	-
3378	-	0.500	-
3371	-	0.500	-
Control	-	0.415	-



(a) (b) (c)

Figure 3.5. Healthy nodules on the roots of a plant infected with strains 620 (b) and 385 (c). The root of uninoculated plant (a) is also shown.

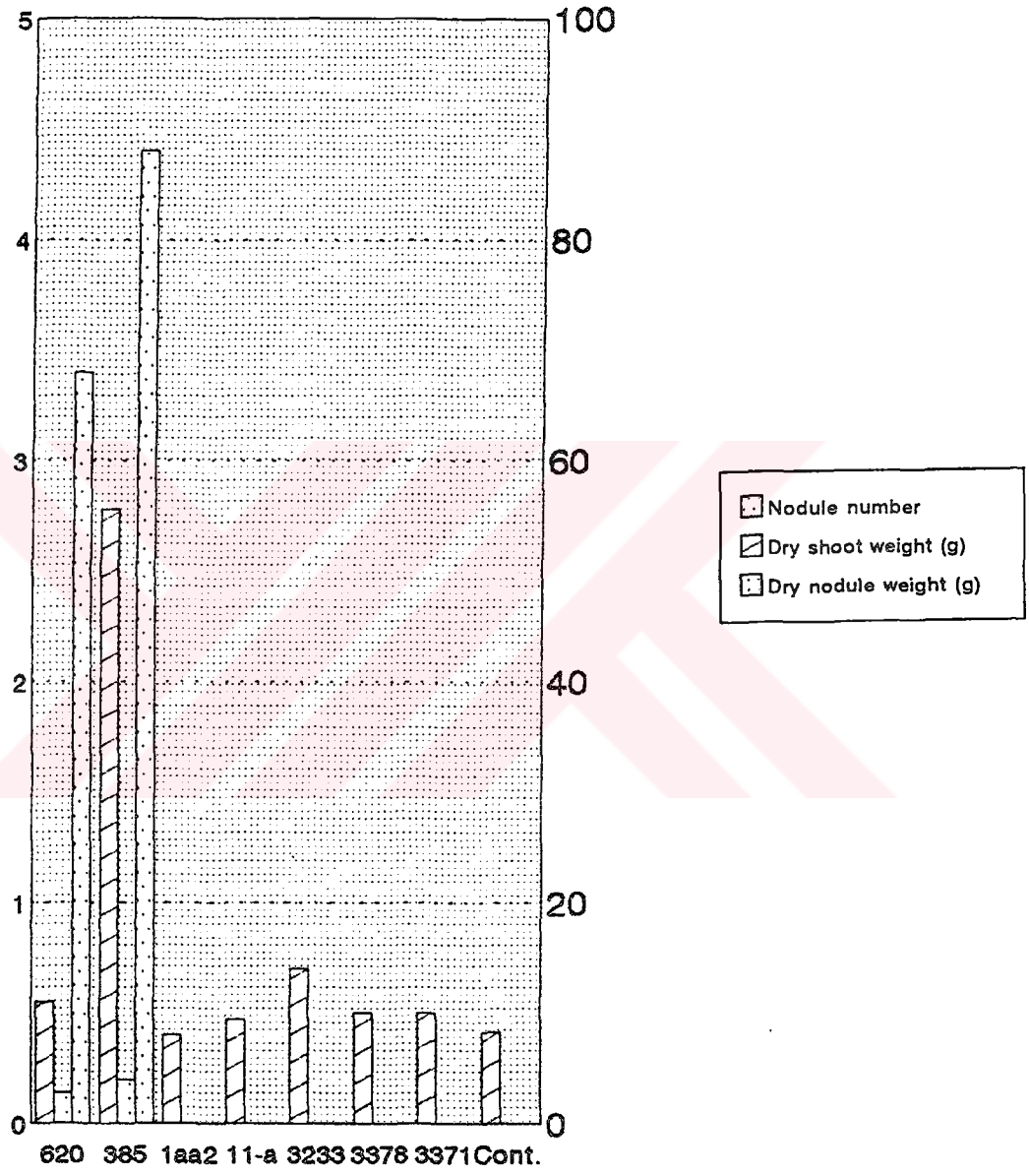


Figure 3.6. Diagramatic representation of the effectiveness of various *R.cicer* strains.

3.6. Plasmid Profiles of Effective Strains

Different procedures have been reported for the extraction of megaplasmids from *Rhizobium* (Rosenberg *et al.*, 1981; Finan *et al.*, 1986; Djordjevic *et al.*, 1983). Almost all of these have been devised for the analysis of *R.meliloti* plasmids. Before attempting to analyze the plasmids of *R.cicer* strains, these experimental protocols were experienced with some megaplasmid harboring standard strains. The standard strains included *A.tumefaciens* LB4440, *R.phaseoli* USDA 193 and *R. leguminosarum* 2517 which contained one or two megaplasmids. Of various protocols tested in our laboratory, Casse and Boucher (1979)'s procedure was the only one by which the plasmids could be successfully isolated. It should be noted that throughout this study, megaplasmid isolation from rhizobia was proven to be rather difficult as it gave nonreproducible results. *R.phaseoli* USDA 193 contained a single plasmid of 200 Kb. *A.tumefaciens* LB4440 also had a single plasmid of unknown size, but smaller than 200 Kb (Figure 3.7). *R.leguminosarum* 2517 was the most useful as a size marker since it contained two plasmids of known sizes; 200 Kb. and 130 Kb, respectively. As shown in Figure 3.8.a, our effective strain 620 gave two plasmid bands of 75.8 Kb. and 46.8 Kb, respectively (Figure 3.8.b).

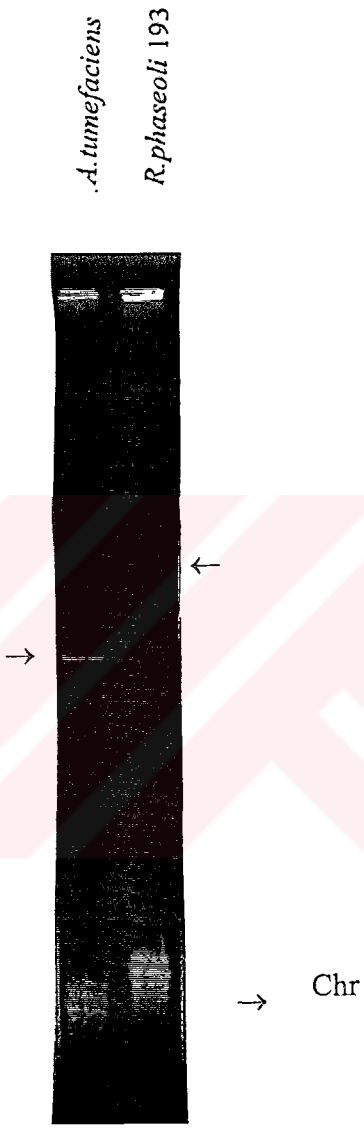


Figure 3.7. Plasmid patterns of *A.tumefaciens* LB4440 and *R.phaseoli* USDA 193.

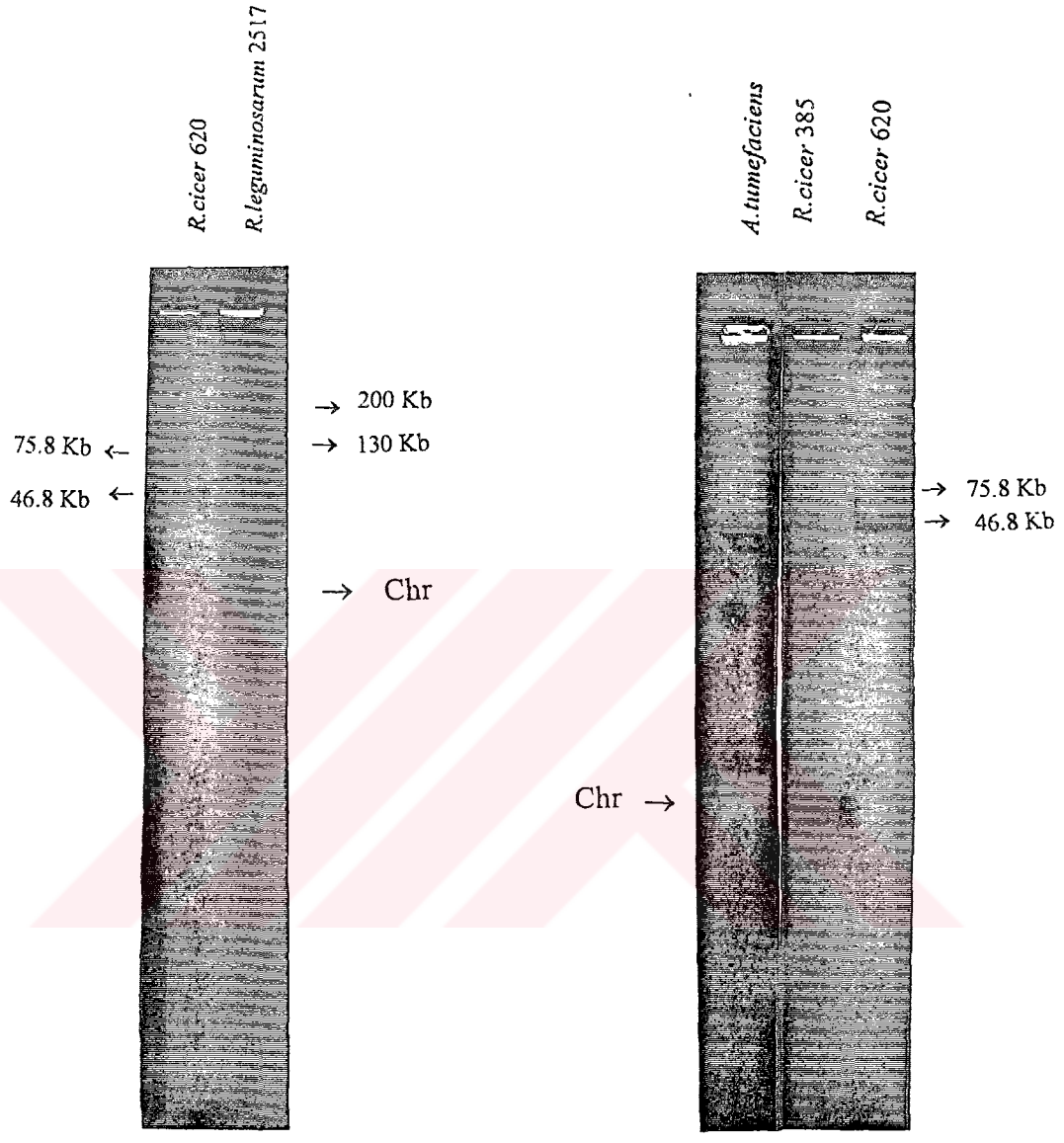


Figure 3.8. Plasmids of *R.cicer* 620 (a) and *R.cicer* 385 (b). The standard strain *R.leguminosarum* 2517 is also included as plasmid size marker.

Plasmids could not be detected in strain 385 while the non-nodulating strain 3233 did have five or six high-molecular weight (316.2 Kb, 200 Kb, 95.5 Kb, 63.0 Kb, 38.0 Kb.) and two low-molecular weight plasmids (Figure 3.9).

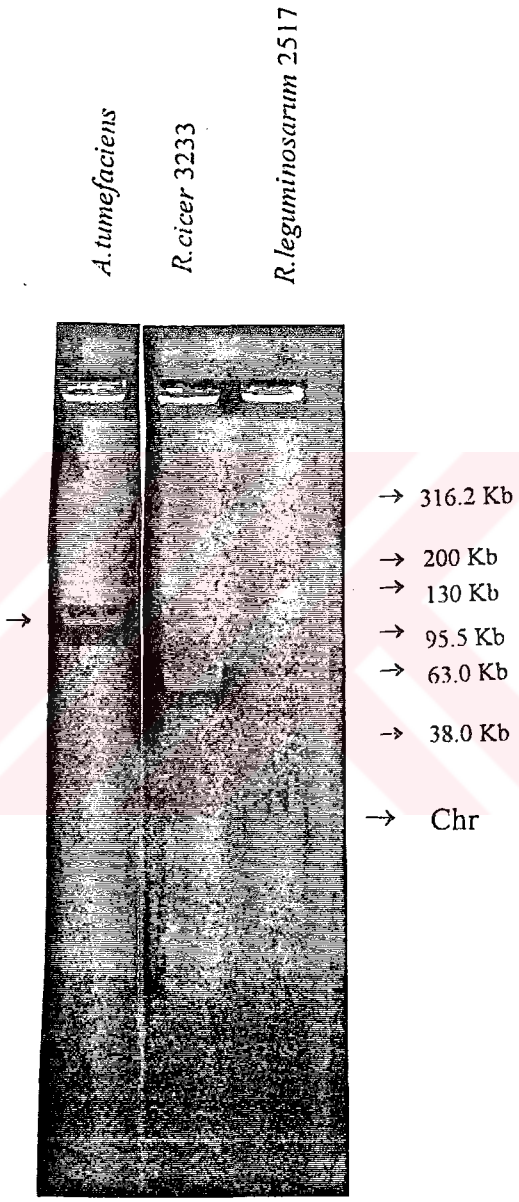


Figure 3.9. Plasmid pattern of the non-nodulating strain 3233.

3.7. Effect of Mixed Inocula

Different combinations of the selected strains were employed to study the effect of multiple infections (Figure 3.10).

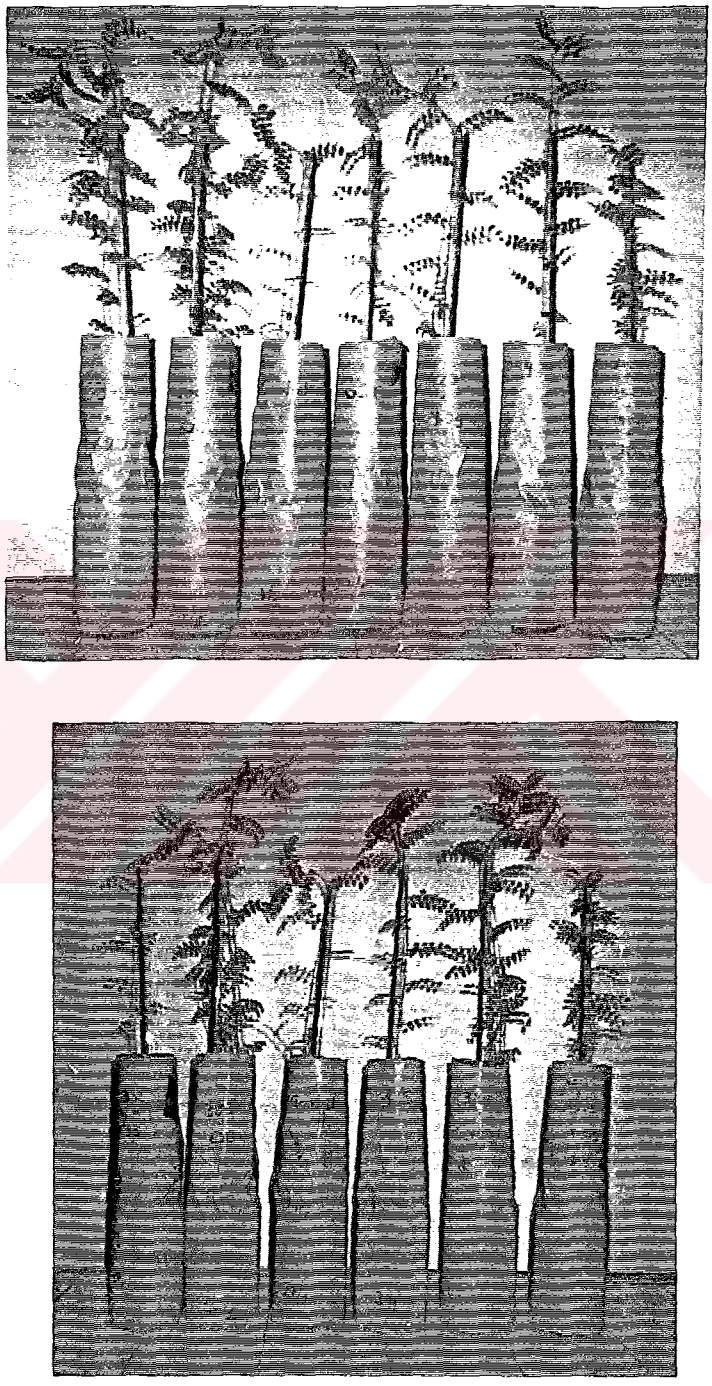


Figure 3.10. Plants grown in Leonard jars.

The use of mixed inocula proved to be much better than the use of individual strains. The infections of Y-29x385 and Y-29x620x3379 resulted in 35 to 31 fold increases in total nitrogen respectively, whereas the increase ranged between 1.5-3.5 fold when single strains were applied (Table 3.4). The use of fertilizer in place of single inoculants, however, was more useful as the increase in total nitrogen and total protein approached to those obtained from multiply infected plants.

Table 3.4. Effect of mixed inocula on nodulation and chickpea yields.

	Nodule number	Shoot dry weight (g)	Shoot dry weight increases (% of control)	Protein (mg/ml)	content increase (fold)	Nitrogen (mg/ml)	content increase (fold)
385	73	0.55	189.47	0.110	1.000	0.0560	03.2
620	58	0.27	42.100	0.140	1.270	0.0560	03.2
3379	75	0.45	136.84	0.117	1.060	0.0245	01.4
Y-29	76	0.55	189.47	0.115	1.045	0.0595	03.4
620x3379	97	0.50	163.15	0.235	2.130	0.4130	23.6
385x3379	47	0.24	26.310	0.115	1.045	0.4690	26.8
620x385	59	0.54	184.21	0.110	1.000	0.4200	24.0
620xY-29	71	0.48	152.63	0.212	1.920	0.4830	27.6
Y-29x3379	53	0.50	163.15	0.130	1.180	0.5180	29.6
Y-29x385	78	0.60	215.78	0.260	2.360	0.6160	35.2
Y-29x620x385	87	0.52	173.68	0.245	2.220	0.0280	01.6
Y-29x620x3379	98	0.59	210.52	0.230	2.090	0.5530	31.6
FERTILIZER	-	0.45	136.84	0.255	2.310	0.5460	31.2
CONTROL	-	0.19	-	0.110	-	0.0170	-

Fertilizer supplemented (plus-nitrogen), uninoculated control and multiply infected plants are shown in Figure 3.11. The remarkable difference in shoot size was not reflected on other parameters of plant growth as nitrogen and protein contents of multiple-infected and fertilizer-applied plants were almost equal.

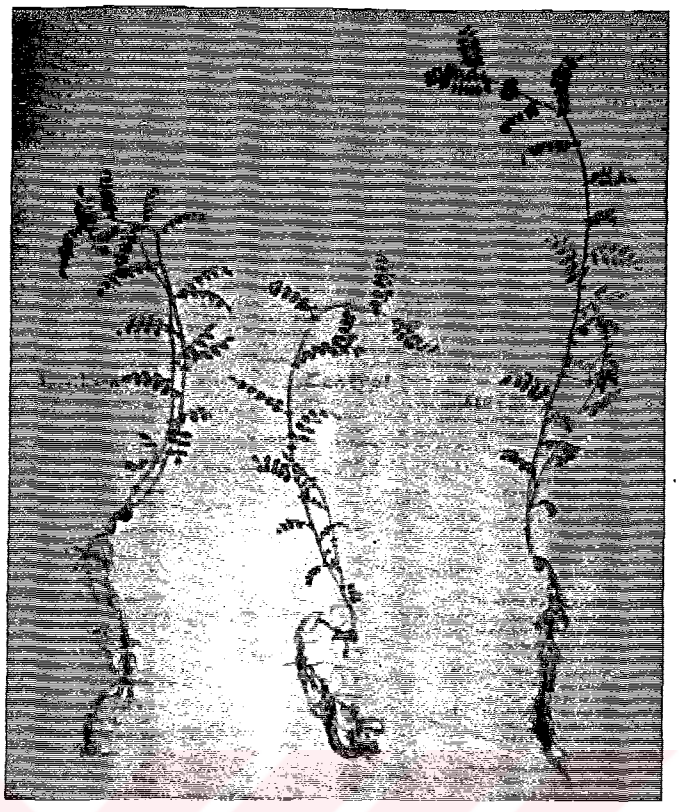


Figure 3.11. Fertilizer-supplemented, uninoculated control, and multiply infected plants just after harvest.

3.8. Competition for Nodulation

3.8.1. Nodule Occupancy

Nodulation competitiveness of the strains was next determined. The strains occupying each nodule were identified according to their antibiotic resistance patterns. The competitiveness of the strains 620 and 385 could not be defined since there was no difference in their antibiotic resistance patterns. The results are tabulated in Table 3.5. Strain 385 induced more root nodules than 3379 and Y-29. Strain 620, on the other hand, was the most competitive as its nodule occupancy was higher than all these strains.

Table 3.5. Nodule occupancy by strains of *R.cicer*.

Combinations	Antibiotic markers	Number of nodules occupied	Nodule Occupancy (%)
620x3379	Str^R 40 Str^S 40	20 nodules 10 nodules	%66.00 620 %34.00 3379
385x3379	$Clox^R$ 75 $Clox^S$ 75	18 nodules 15 nodules	%54.50 385 %45.50 3379
620xY-29	Spc^R 100 Spc^S 100	13 nodules 21 nodules	%38.20 Y-29 %61.80 620
Y-29x385	Amp^R 75 Amp^S 75	5 nodules 28 nodules	%84.84 385 %15.15 Y-29
Y-29x3379	Amp^R 75 Amp^S 75	12 nodules 4 nodules	%75.00 Y-29 %25.00 3379

^{S}, sensitive; ^{R}, resistant

3.8.2. Rhizospheric Colonization

Rhizosphere colonization which is the other component of competitiveness was also assessed. As was also seen in nodulation competitiveness experiments, 3379 was the least competitive and 385 was slightly better (Table 3.6). Strain Y-29 appeared to be the most competitive organism in terms of rhizosphere occupancy while 620 was the best in inducing root nodules.

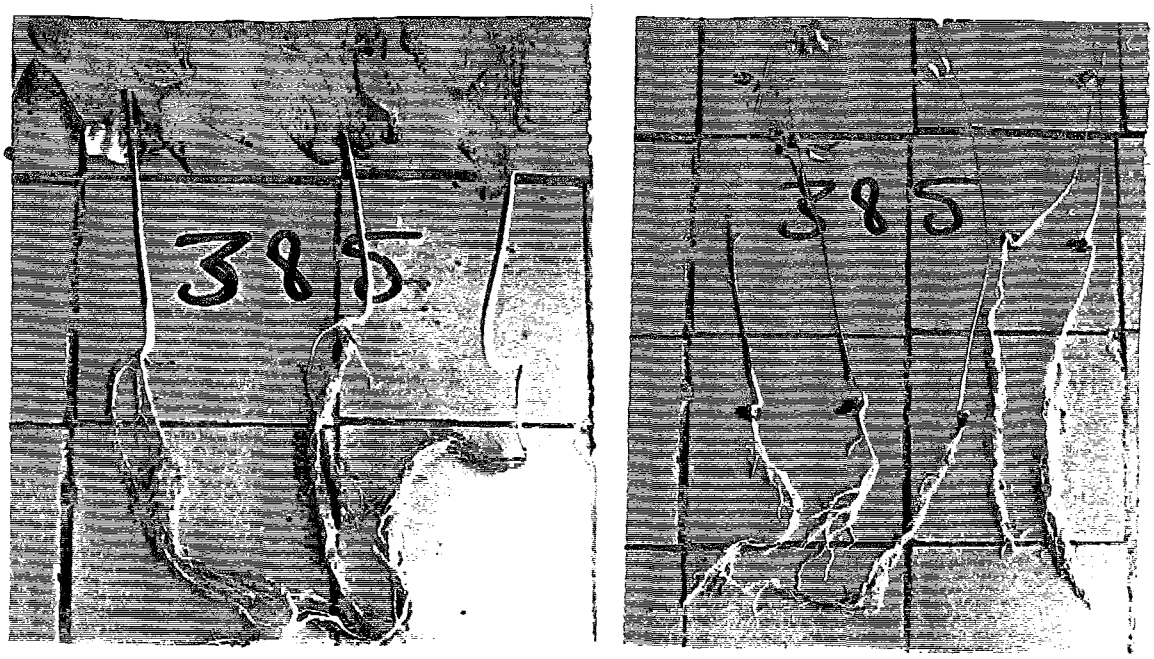
Table 3.6. Rhizospheric colonization of strains of *R.cicer*.

Combination	Antibiotic markers	Colony forming units/ml	Average Rhizosphere colonization (%)
620xY-29	Master plate Str^R 40 Spc^R 100	$5.2x10^6$ $2.8x10^6$ $3.2x10^6$	57.400 (Y-29)
3379xY-29	Master plate Amp^R 75 Chl^R 30	$4.8x10^5$ $4.2x10^5$ $2.7x10^5$	71.875 (Y-29)
620x3379	Master plate Str^R 40 $Clox^R$ 75	$1.6x10^5$ $8.0x10^4$ $1.2x10^5$	62.500 (620)
Y-29x385	Master plate Amp^R 75 Spc^R 100	$2.1x10^6$ $1.7x10^6$ $1.3x10^6$	71.400 (Y-29)
385x3379	Master plate $Clox^R$ 75 Lin^R 50	$3.5x10^5$ $2.4x10^5$ $2.1x10^5$	64.250 (385)

^{R}, resistant

3.9. Host Specificity

To determine the host range of *R.cicer* 620 and 385, the strains were used as inoculants for bean (*Phaseolus vulgaris*) and lentil (*Lens culinaris*). The strains did not infect these plant species and could not produce any nodule (Figure 3.12).



Phaseolus *Lens*

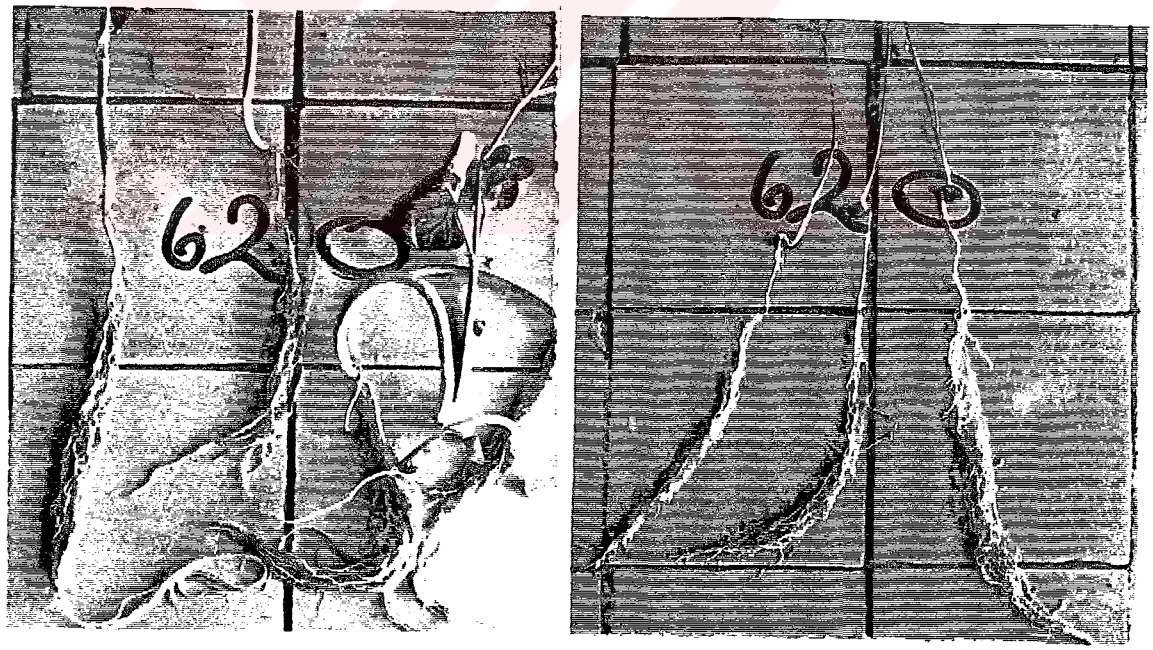


Figure 3.12. The roots of *phaseolus* and *lens* infected by strains 385 and 620

3.10. Effect of Inoculum size

To determine the effect of inoculum size, chickpea seeds were inoculated with different concentrations of bacteria (10^6, 10^7, 10^8, 10^9 cells/ml). The results of this experiment are presented in Table 3.7.

Table 3.7. Effect of inoculum size on symbiotic effectiveness

Strain	Inoculum size	Nodule number	plant shoot dry weight (g)	Nodule dry weight (g)
R.cicer	10^6	22	0.280	0.020
	10^7	27	0.378	0.037
620	10^8	31	0.345	0.040
	10^9	45	0.446	0.060
R.cicer	10^6	21	0.280	0.042
	10^7	24	0.376	0.054
385	10^8	27	0.318	0.051
	10^9	31	0.516	0.058

For both strains, as the inoculum size increased, the nodule number also increased with correspondingly high shoot dry weights.

3.11. Effect of pH on Bacterial Growth and Nodulation

Before attempting to determine the effect of pH on symbiotic capacity, the growth of *R.cicer* strains under different pH's was estimated. The extent of growth was determined in a series of YEM cultures each of which did have a different initial pH within a range of pH 5.0 to 9.0. Before determining the growth, the final pH of each medium was measured. Both strains were found to show an acid reaction, thus decreasing the pH of media. The strain 620 grew best in the medium adjusted to pH 7.0 while 385 displayed its optimum growth when the pH was 8.0 (Table 3.8).

Table 3.8. Effect of pH on bacterial growth

	pH of the medium		Bacterial growth	
Strain	Initial	Final	A_{600}	CFU/ml
R.cicer	5	5.87	0.187	$1.45x10^4$
	6	7.06	0.204	$2.25x10^4$
620	7	6.72	0.563	$4.75x10^6$
	8	7.54	0.630	$7.50x10^5$
	9	7.32	0.218	$6.88x10^4$
R.cicer	5	5.18	0.185	$3.50x10^2$
	6	5.87	0.384	$5.25x10^4$
385	7	6.57	0.398	$1.50x10^5$
	8	7.01	0.426	$5.88x10^5$
	9	7.19	0.408	$2.75x10^4$

Our results concerning the effect of soil pH on nodulation by these strains are tabulated in Table 3.9.

Table 3.9. Effect of pH on symbiotic effectiveness

Strain	pH	Nodule number	Shoot dry weight (g)	Nodule dry weight (g)
R.cicer	5	32	0.17	0.04
620	6	34	0.28	0.08
	7	53	0.33	0.1
	8	71	0.34	0.12
	9	27	0.38	0.07
R.cicer	5	19	0.24	0.06
385	6	16	0.3	0.03
	7	25	0.33	0.11
	8	27	0.32	0.06
	9	37	0.31	0.07

Strain 620 showed the highest performance when it was inoculated to seeds grown in soils of pH 8.0. However, the results obtained from strain 385 was rather conflicting. This strain exerted highest contribution to chickpea yields at pH 7.0 as reflected by shoot and nodule dry weights whereas it produced more nodules in soils adjusted to an alkaline pH (9.0).

3.12. Effect of Mineral Levels on Nodulation

To study the effect of calcium, phosphate and ferric iron, Jensen media in Leonard jars were supplemented with various concentrations of these minerals (Table 3.10).

Table 3.10. Effect of minerals on symbiotic effectiveness when used at different concentrations.

		R.cicer 385			*R.cicer* 620		
Minerals	Conc. (mM)	Nodule number	Shoot dry weight (g)	Nodule dry weight (g)	Nodule number	Shoot dry weight (g)	Nodule dry weight (g)
Ca^{2+}	0.725	39	0.24	0.16	25	0.27	0.062
	1.45 *	31	0.21	0.09	27	0.26	0.074
	2.90	30	0.27	0.04	37	0.31	0.083
	5.8	33	0.29	0.09	42	0.32	0.098
PO_4^{2-}	1.44	32	0.36	0.09	42	0.3	0.15
	2.88 *	25	0.28	0.073	27	0.26	0.09
	5.76	21	0.21	0.068	15	0.18	0.05
	11.52	26	0.2	0.01	11	0.14	0.02
Fe^{3+}	0	34	0.18	0.05	33	0.24	0.07
	0.12 *	24	0.21	0.09	27	0.26	0.09
	0.18	20	0.25	0.08	24	0.22	0.03
	0.24	11	0.14	0.02	21	0.22	0.02

* the amounts routinely incorporated into Jensen medium

Phosphate is routinely incorporated into Jensen medium at a final concentration of 2.88 mM. All the criteria of symbiotic effectiveness increased when this concentration was halved. Two and four fold increments in this concentration on the other hand, resulted in a general decrease in all criteria.

In strain 620 the number of nodules and plant mass were found to increase with increasing Ca^{2+} concentration. In strain 385, however, there was an inverse relationship between nodulation and Ca^{2+} levels.

For both strains, ferric iron was not necessary for nodulation to occur since the number of nodules even increased upon the omission of this mineral. On the other hand, shoot and nodule dry weights slightly increased by the inclusion of iron at 0.12 mM to 0.18mM concentrations.

3.13. Nodulation of Chickpea in Agar Tubes

The culturing of chickpea in agar tubes was also tried in this work with the aim of obtaining a less laborous experimental set up for future nodulation studies. Using Jensen agar tubes containing air bubbles, nodulation of chickpea was obtained in 280x25 mm tubes containing 35 ml agar medium. In experiments with batches of 5 air-trapped tubes for each strain and 3 tubes for uninoculated control, however, positive results were not evident in all the inoculated tubes. Nodulation was detected only in two of the tubes which have been inoculated with strain 620 (Figure 3.13). In these tubes, there were markedly more profuse root growth as well as higher shoot mass as compared to the control plants (Table 3.11).

Table 3.11. Nodulation in agar tubes

	Uninoculated control	Tube inoculated with *R.cicer* 620
Nodule number	-	7
Shoot dry weight (g)	0.07	0.12
Nodule dry weight (g)	-	0.01

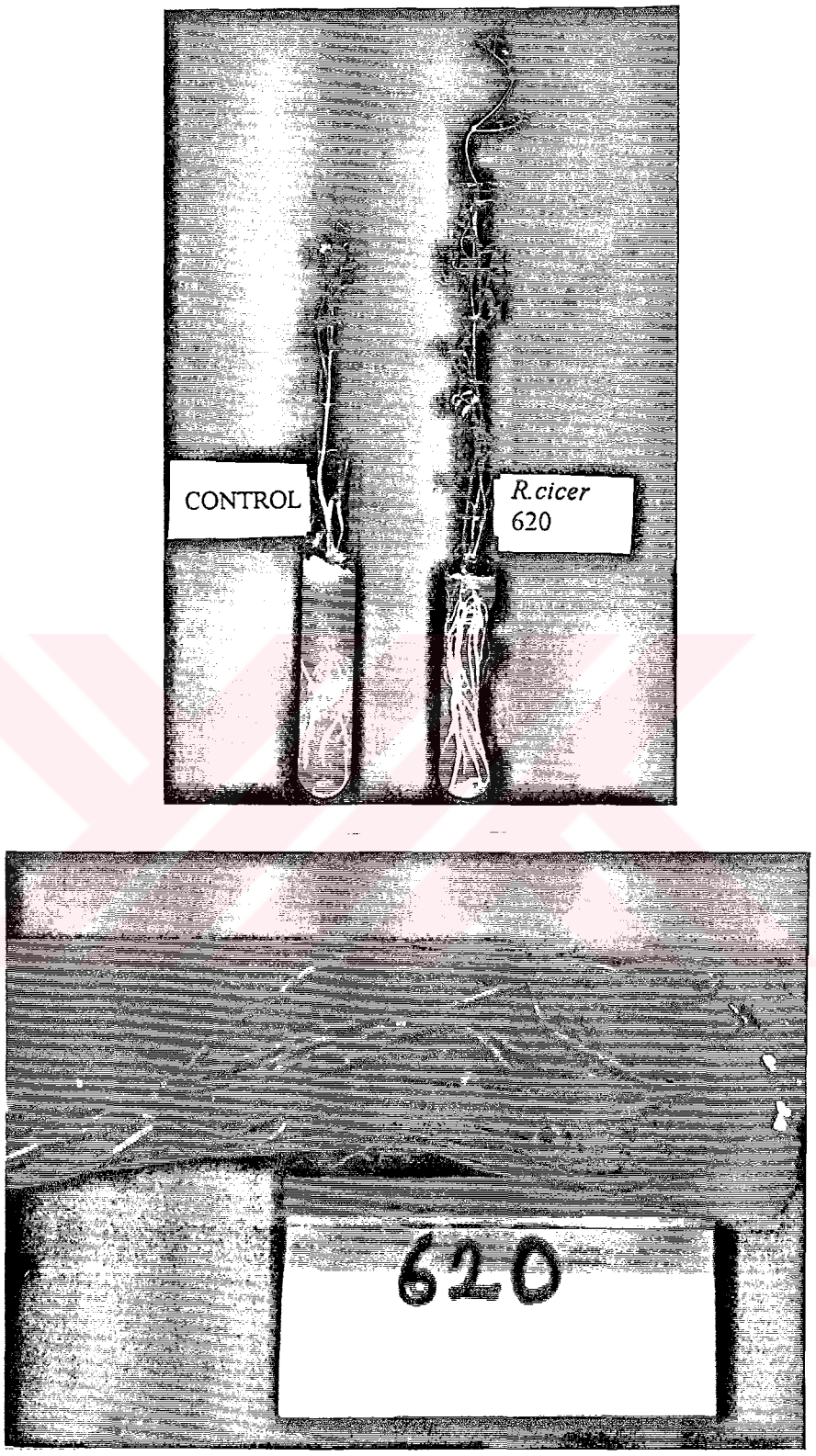


Figure 3.13. Nodulation of chickpea roots in 0.8 % agar tubes.

3.14. Broadening the Host Range

Aiming at broadening the host range of the most effective strain *R.cicer* 620, it was transformed with *R.leguminosarum* T3 (ura, trp, str) harboring four distinct high molecular weight plasmids. One of these plasmids which has been designated pJB5J1 (pRL1J1:: Tn 5) (kan^r) was a nodulation plasmid conferring the specificity for pea (Brewin *et al.*, 1980b). The transformants were selected on Kan-60 plates. Of many Kan^R colonies developed on selective plates, three were kept and analyzed for their plasmid contents. Interestingly, these derivatives were found to lack the plasmids of both the recipient strain 620 and donor T3 (Figure 3.14).

Figure 3.14. Plasmid profiles of 620, T3 and the transformant strain.

3.15. Statistical Analysis of Data

For better understanding of the results of plant experiments, the numerical data regarding nodule number, shoot dry weight and nodule dry weight were analyzed using a SPSS computation program. The correlation coefficients thus computed are presented in Table 3.12.

Table 3.12. Correlation coefficients for the variables tested.

	Correlation Coefficients [a]					
	R.cicer 620			*R.cicer* 385		
	Nodule number	Shoot dry weight	Nodule dry weight	Nodule number	Shoot dry weight	Nodule dry weight
Inoculum size	0.8159 **	0.3545	0.6050**	0.5336 *	0.5614 *	0.2823
pH	0.2331	0.9378 *	0.5213	0.9134 *	0.7155	0.2744
Ca^{2+}	0.9540 *	0.8778	0.9739 *	-0.3673	0.8383	-0.4475
PO_4^{2-}	-0.8672	-0.9436	-0.9053	-0.3790	-0.8391	-0.9770 *
Fe^{3+}	-1.0000	-0.5606	-0.7236	-0.9877 *	-0.1048	-0.2469

a: * Significant at a level of 0.5 %

** Significant at a level of 0.1 %

The nodule number should be the index of choice for symbiotic effectiveness as it correlated well with most of the parameters tested. Giving the highest significant correlation in more than one index in both strains, the inoculum size was the most effective variable.

CHAPTER IV

DISCUSSION

Efficient growth of agricultural crops depends upon an adequate supply of usable nitrogen. This may be provided by nitrogenous fertilizers or through microbial reduction of atmospheric nitrogen. In order to reduce reliance on chemical fertilizers, attention has focused on increasing the supply of biologically fixed nitrogen. However, large amounts of energy are required for biological nitrogen fixation and thus the amount of nitrogen fixed is related to the accessibility of available energy sources. Accordingly, free-living nitrogen fixers (with the exception of the photosynthetic prokaryotes) do not normally fix large amounts of nitrogen. The most important nitrogen fixing associations in nature are symbiotic and involve in particular *Rhizobium* with leguminous plants.

Plant-microbe interactions in the rhizosphere, although poorly understood, have crucial effects on plant growth (Long, 1989). Thousands of soil bacteria and other microorganisms live in the rhizosphere and utilize the plant as a source of nutrients, thus forming highly complex relationships. The magnitude of these interactions is usually regulated by the environment in which the organisms are functioning.

In this study, the available strains (5 standard and 2 local) of *R.cicer* were evaluated for their ability to nodulate chickpea (*Cicer arietinum*). Only two of them,

designated 620 and 385, could form nodules on the roots. The nodules were considered healthy based on their pink color and microscopic examination of bacteroid forms from nodule extracts. If the inoculated plants form nodules and produce healthy green leaves when grown in nitrogen-free media, it might be assumed that a symbiosis has been established (Somasegaran and Hoben, 1985). However, final evaluation of the symbiosis should be based on several measurable parameters such as shoot dry weight of plant harvested, nodule number and nodule dry weight. In the light of these parameters, strain 385 proved to be more effective than 620.

The term 'bacteriocin' refers to any inhibitory agent which causes antagonism between closely-related strains and which is neither self-propagating (i.e. bacteriophage) nor an antibiotic with activity against a wide range of microorganisms (Emerich and Wall, 1985). Many bacteriocins of enterobacteria are plasmid-determined (Hardy, 1975) and this may be a feature common to other bacterial genera. Bacteriocin production by the species of *Rhizobium* has been reported (Roslycky, 1967; Schwinghamer and Bekangren, 1968; Schwinghamer, 1971; 1975; Lotz and Mayer, 1972; Schwinghamer *et al.*, 1973). In a study which involves an investigation of bacteriocin production by 97 isolates of *R.leguminosarum* (Hirsch, 1978), two types of bacteriocins were identified and designated small and medium on the basis of their size. High frequency conjugal transfer of the ability to produce medium-sized bacteriocins from three different *R.leguminosarum* field isolates to other strains of *R.leguminosarum*, suggested the presence of bacteriocinogenic plasmid in these strains. Although no transfer of the ability to produce small-bacteriocins was detected, the concominant loss of production and resistance on the introduction of plasmids determining medium-bacteriocin production implied that these properties could be determined by an

incompatible plasmid. Although bactriocin production is highly common among *Rhizobium* spp., none of our strains produced such inhibitory substances.

Antibiotic resistant strains of *Rhizobium* were identified by their ability to grow on a series of media containing different antibiotics. The antibiotic marker technique finds application in ecological studies of *Rhizobium* where strain identification is not possible by serology due to cross reactions of the strains or because of unavailability of antisera (Somasegaran and Hoben, 1985).

Sensitivity to aminoglycoside antibiotics other than streptomycin was common among *R.cicer* strains used in our study. This did not accord well with the previous reports (Josey et al., 1979) which described the widespread resistance to aminoglycosides among various species of *Rhizobium*. It must be noted that our effective strains (385, 620 and Y-29) were resistant to streptomycin. Our strains varied greatly in terms of their susceptibilities to other antibiotics such as lincomycin, chloramphenicol, cloxacillin, tetracycline and ampicillin. Streptomycin-resistance is frequently used as a marker characteristic for rhizobia. Mutants resistant to this aminoglycoside antibiotic have been reported to be stable, have a low incidence of cross-resistance and lose their symbiotic capacity only infrequently. The resistance to spectinomycin and rifampicin have also been used as markers. Highly resistant mutants with single or double markers (Streptomycin-spectinomycin or streptomycin-rifampicin) can be obtained with one exposure to low concentrations of these antibiotics (Somasegaran and Hoben, 1985).

Different combinations of effective strains were next studied to determine the effect of multiple infections. The mixed inocula contained the strains at a 1:1 ratio. The double infection of Y-29 and 385 as well as the triple infection of Y-29, 620 and

3379 gave rise to the maximum values for all parameters tested. In mixed infections the number of nodules formed by each strain generally depends not only on the nodulation competitiveness of the strains but also on their numbers. To evaluate the performance of one strain in relation to another, one must know the relative numbers of these strains in the inoculum as well as the proportion of nodules occupied by them (Boonkerd *et al.*, 1978; Amarger and Lobream, 1982).

We studied rhizospheric colonization and interstrain competition for nodule occupancy by 4 effective inoculum strains (620, 385, 3379, Y-29). The inoculum strains markedly differed in their ability to induce root nodules in the presence of another inoculum strain. The use of antibiotic resistance patterns in competition studies has been introduced by Van Rensberg and Strijdom, (1982). This approach was proven to be very successful in our study. Strain 620 occupied the greatest number of nodules in all double infection tests. The strain 385 was also competitive as it occupied relatively more nodules than either Y-29 and 3379. The competitiveness of strains 620 and 385 could not be compared because of the inavailability of the necessary antibiotic markers. As pointed by Abaidoo *et al.* (1990) the competition pattern (the proportion of nodules formed by each of the four strains) was a stable character and was not related to rhizospheric colonization. Though strain Y-29 displayed highest rhizospheric colonization in all double infection tests, it was not as competitive as 620 and 385 in its nodule occupancy. The factors that contribute to the competitiveness include motility (Ames and Bergman, 1981; Mellor *et al.*, 1987), cell surface polysaccharides (Handelsman *et al.*, 1984; Ugalde *et al.*, 1986) and bacteriocin production (Triplett and Barta, 1987) although the importance of these factors for competitiveness in the field has not been demonstrated. It can be noted at this point that the strain 3379 which was the least competitive in both rhizosperic colonization and nodule occupancy did not form

typical gummy colonies and appeared to be exopolysaccharide-negative. The results obtained under defined laboratory conditions must be interpreted with caution, as different results may be obtained in field tests (Van Rensberg and Strijdom, 1982). Thus, even when the strains are strongly competitive, the environmental factors may decide which strains will predominate in nodules. Environmental parameters of possible significance in field situations include host genotype, microbial antagonism, soil factors and climatic conditions (Ham *et al.*, 1971; Graham *et al.*, 1980).

The inoculum level is an important parameter in field applications especially when there is an indigenous *Rhizobium* population in soil. Our results showed that as the inoculum size increased, there was a corresponding increase in nodule occupancy by the introduced strains (620 and 385). This was the general trend obtained in our study. Beattie *et al.* (1989) defined the relative nodulation competitiveness of two strains as the relationship between the proportional representation of the strains in the inoculum and the proportional of the representation of the nodules occupied by each strain. Thus, a given strain is a successful competitor against a second strain if the proportion of the nodules it occupies is greater than its proportional successful representation in the inoculum. Somasegaran *et al*,. (1988) demonstrated that the competitive abilities of the effective strains (TAL 1148 and TAL 620) or ineffective strain (TAL 480) were not influenced by inoculum size. In a field study, various isolates of *R.trifolii* have been evaluated by their nodule occupancy as a function of inoculum concentration (Martenson, 1990). The isolates nodule occupancy of which was not correlated with inoculum concentration were selected as the highly competitive ones. When plants were multiply infected, such strains were present in the largest number of nodules.

Statistical analysis of data obtained from our experiments yielded correlation coefficients which were significant at both levels, indicating that inoculum size is a very important variable in determining the success of symbiosis.

The effect of soil pH on nodulation by *R.cicer* 620 and 385 was also investigated. Although it has been well documented that low pH inhibits nodulation by *R.leguminosarum*, *R.phaseoli* and *Bradyrhizobium* spp. (Franco and Munns, 1982; Evans *et al.*, 1980; Hohenberg and Munns, 1984), *R.cicer* strains used in our study were fairly resistant to low pH as they nodulated the plants even under acidic conditions. The optimum nodulation, on the other hand, appeared to occur between pH 7.0 to 8.0. Bromfield and Jones (1980) have pointed to a positive correlation between final pH of the culture medium and symbiotic effectiveness. The effect of soil acidity on the outcome of symbiosis of peas and broadbeans with *R.leguminosarum* strains was reported by Haktanir and Göktan (1979) in greenhouse experiments. They found that the number of viable Rhizobia decreased by time when they were introduced to acid soils (pH 4.85 and 5.20). Our strains tended to neutralize the pH of the medium when grown freely in YEM broth adjusted to different pH values. This might account for the success in establishment of symbiosis in both acidic and alkaline conditions. While the results of our experiments suggested such aspects of pH effect on *R.cicer* strains, correlation coefficients computed did not indicate a significant effect of soil pH on the outcome of symbiosis.

In regard to the survival of rhizobia in acidic soils, Caetano-Anolles *et al.* (1985) have postulated that the survival is inversely related to the amount of acid produced, that is, those rhizobia producing the least acid should survive in acid soils. Acid production, on the other hand, depends on the available substrates even among

slow-growing rhizobia which are normally considered to increase the pH of the medium (Franco and Munns, 1982; Tan and Broughton, 1981).

The mineral requirements of high plants have been studied in great detail (Marschner, 1986). Several criteria have been proposed for a mineral nutrient to be considered essential. The plant must not be able to complete its life cycle in the absence of the mineral element, the mineral must not be replaceable with another element, and it must be involved in some component of plant metabolism, e.g. as part of an enzyme cofactor.

Several criteria have also been proposed to assess the mineral constraints inherent to the symbiotic nitrogen fixation. There should be a higher requirement for nutrients in symbiotic nitrogen fixation than that for the growth of the host plant alone (O'Hara *et al.*, 1988a). Furthermore, when the symbiotic legume is deficient in the mineral, the addition of combined nitrogen does not improve the development, growth, or nitrogen-fixing capability of the symbiosis.

Growth of *Rhizobium* in pure cultures under low phosphate levels has been found to vary among strains (Beck and Munns, 1984), thus the levels of the available phosphate in soil should influence the symbiosis not only because this mineral is essential for the plant, but it also affects the growth of the bacterial counterpart. Furthermore, nitrogen fixation imposes a considerable energy drain on the energy metabolism. *In vivo* nitrogen fixing cells have ADP/ATP ratios of 0.3-0.5, whereas organisms under non-nitrogen-fixing conditions have ratios of 0.8-0.9 (Emerich and Wall, 1985). As opposed to our expectation, increased phosphate concentrations did not result in increased plant productivity, but did have an adverse effect on nodulation and plant yields.

Calcium content of soil resides among the environmental factors that are known to influence plant growth. An excess of calcium is not desired especially because it binds phosphates to form calcium appetite derivatives, thus deprives the plant from this essential mineral. Although the effect of calcium on *Rhizobium*-legume symbiosis has not been well-documented, this mineral was shown to affect *nod* gene induction indirectly (Richardson *et al.*, 1988). It was found in our study that the increment in calcium levels did not much alter the symbiotic effectiveness of strain 385 while it did exert a positive effect on symbiosis with strain 620.

It has been demonstrated that the application of iron to iron- stressed legumes increases the nodule number and plant mass (Rai *et al.*, 1982). This observation does not indicate whether the application of iron has an indirect effect on the iron metabolism of the plant or on the development of functional nodules. It has been shown that iron deficiency in peanut plants (*Arachis hypogoea*) specially limited bradyrhizobial nodule development and the development of a functional nitrogen-fixing apparatus (O'Hara *et al.*, 1988b). There is a wide variation among plants in their ability to acquire iron from their natural environment and accordingly they are termed as either iron-efficient or -inefficient. In our study, ferric iron was not necessary for nodulation to occur and nodule formation was even more effective when this metal was omitted from the medium. In spite of this, plant and nodule masses did not accordingly increase in such iron-free environments. It appears that the nodules formed in the absence of iron were not much active in nitrogen fixation, hence did not contribute to the plant yields.

Various methods are available for culturing legumes to carry out nodulation studies and to assess efficiency of rhizobia. They include the use of test tubes with

seedling agar (Dart, 1977), vials containing vermiculate (Wacek and Brill, 1976), Leonard Jar (Somasegaran and Hoben, 1985) and Chilum jar assemblies (Dahiya and Khurana, 1981) containing washed sand, and pots containing soil sand or perlite (Vincent, 1970). Of these, the method of nodulation in agar tubes carries advantages such as asepsis, economy of space, convenience in handling and easy availability of agar. However, this method has been useful only with small-seeded legumes; large-seeded ones (such as chickpea) show little or poor nodulation in agar tubes. To solve this problem, Lakshminarayana *et al.* (1988) adopted a simple technique in which numerous air bubbles were trapped in agar deeps during their preparation. The success in obtaining well-nodulated chickpea and soybean in this system was attributed by the authors to (i) making the medium more porous and soft, enabling better root penetration and growth, (ii) providing more aeration to both the growing roots and the rhizobia, thereby enabling better survival of rhizobia, and (iii) favoring appearance of more root hairs leading to infection by rhizobia. The feasibility of this technique was tested in our laboratory by using strains 620 and 385 as the inoculants. In this system, the shoots were allowed to grow for two weeks. Visible nodules with considerably higher shoot weights were formed only in tubes inoculated with strain 620. It follows that the success of this method might also be determined by the properties of the inoculant used. Although it permits a direct examination of root system in agar tubes, we do not believe that it could be of a considerable utility in rapid identification of the nodulation capabilities of soil isolates.

Nodulation by fast growing rhizobia has been mostly correlated with the presence of large plasmids (Pimental, 1975; Casse and Boucher, 1979; Beringer, 1973; Banfalvi *et al.*, 1981; Long, 1984). In several strains of *R.leguminosarum*, the identified genes (*nod* ABC) necessary for root nodulation have been found to be located on specific large plasmids (Djordjevic *et al.*, 1985; Innes *et al.*, 1985; Innes *et*

al., 1988). In the *R.meliloti* strains studied thus far, the *nif* genes that have been mapped are located on a symbiotic megaplasmid. Mutations that map in the *R.meliloti* chromosome or to a second megaplasmid have been shown to result in *Fix*⁻ phenotypes (Ligon, 1990), indicating that some genes required for symbiotic nitrogen fixation may be located there. Unlike the fast-growing rhizobia, the *nif* and *fix* genes of *Bradyrhizobium japonicum* are located on the chromosome (Lamb and Hennecke, 1986; Scott, 1986).

In the present study, it was found that *R.cicer* 620 harbors two high molecular weight plasmids. This suggested the possible involvement of one or both plasmids in nodulation and/or symbiotic nitrogen fixation. Plasmid curing and conjugal transfer studies are necessary to establish such a correlation. On the other hand, the related molecular studies could not be successfully performed because of the difficulties encountered during the isolation of megaplasmids. Plasmid patterns of the strains showed some variations from experiment to experiment, most probably resulting from an interference by high polysaccharide content of the cell surfaces or the membrane-bound nature of the plasmids. The growth of bacteria in sugar-free media as well as the successive salt washing of the cells prior to extraction contributed a little, and a more effective procedure is required to avoid conflicting results in plasmid analyses. Besides, one should also have a more convenient and practical system for the cultivation of plants which would also permit an easier and much rapid assessment of root nodulation and plant productivity.

Certain conjugative plasmids of *R.leguminosarum* have been shown to confer the ability to nodulate peas on several species of *Rhizobium*, including *R.phaseoli* and *R.trifolii* (Beynon *et al.*, 1980). Incompatibility has also been observed between *Rhizobium* plasmids. Some of the transconjugants isolated have lost information

required to nodulate the original host (Beynon *et al.*, 1980; Brewin *et al.*, 1980b), it has therefore appeared that the presence in the same *Rhizobium* strain of genetic information needed to nodulate host plants of different cross-inoculation groups might cause it to be impaired in its nodulation on either host. In our study, *R.cicer* was transformed with *R.leguminosarum* T3 harboring plasmid pJB5J1 which carries the genes that determine the ability to nodulate peas. Plasmid pJB5J1 is a derivative of the plasmid pRL1J1 of a molecular weight of $130x10^6$ into which the transposon Tn5 (specifying kanamycin resistance) has been introduced (Hirsch *et al.*, 1980). The colonies grown on kanamycin plates were identical in their morphology to strain 620. When their plasmids were extracted, however, we could not show any plasmids. The properties of them might have been somehow altered via transformation or alternatively a possible co-integration of plasmids might have occurred thus increasing the size of resident plasmids and rendering their isolation impossible by the employed method. Incompatibility, on the other hand, is unlikely as the derivatives did contain the plasmids of neither the recipient, nor the donor.

The progress in investigation of symbiotic relationships between plants and soil microorganisms will open new ways to control soil-borne diseases and to increase crop yields. While one approach involves the selection of strains of beneficial bacteria, the other is to find better ways to coat selected strains of bacteria onto the seeds of important crop plants, hence to obtain cost-effective increases in plant production. Seed coating or dressing is an alternative method of seed inoculation in which the rhizobia are immobilized into a biodegradable polymer support. The formation of a pellet to protect the inoculum from the toxic effects due to acid soil or fertilizer was first developed by coating the uninoculated seed with adhesive and rolling it in finely divided lime (Vincent, 1970). Acacia and gelatin stickers, arabic gum and substituted celluloses have been the supports employed so far (Townshend, 1989; Callon *et al.*,

1990). To date, most attention has focused on liquid inoculation and application are necessary to exploit immobilized inoculant systems.

The results of the present study, overall, indicated the potential applicability of *R.cicer* strains 385, 620 and Y-29 as the inoculants for chickpea, an economically important crop in our country. Multiple infections with these strains gave much better results than the respective single infections, leading to a more effective nodulation as well as a marked increase in nitrogen content. These laboratory findings , however, remain to be supported by field studies. Although growth chamber, light-room and greenhouse experiments are valuable for the primary assessment of the symbiotic capacity of particular rhizobium-host combinations, the full evaluation depends on the field trial. As discussed before, environmental factors directly influence both plant and rhizobia and thus affect the outcome of symbiosis. Biological factors, especially the indigenous rhizobia must also be taken into consideration. The inoculum rate (the amount of inoculant per legume seed) and the abundance in the soil of indigenous rhizobia are expected to determine the success of the inoculant. The ability of an inoculant to adapt to prevailing soil conditions and to persist into subsequent growing seasons is another important factor since it will otherwise be replaced by the indigenous population. The antibiotic resistance markers of our strains identified in this study enable us to track them in related field applications.

CHAPTER V

CONCLUSION

1. Of various local and standard strains of *Rhizobium cicer* screened, strain 385 established the most effective symbiotic association with chickpea.

2. The strains were specific only for chickpea.

3. Nodulation and nitrogen fixation markedly improved by the use of different combinations of effective strains rather than the single infections.

4. The antibiotic resistance markers of strains were successfully used in interstrain competition studies which involved the determination of the proportions of nodules formed by each strain as well as the relative rhizospheric colonization of them. Y-29 displayed highest rhizospheric colonization in all combinations, however it was not as competitive as 620 and 385 in its nodule occupancy. The proportion of nodules formed by each of the strains was therefore not related to rhizospheric colonization.

5. Nodulation genes in fast-growing rhizobia have been reported to be located on specific large plasmids. However, strain 385 did not contain any plasmids while nonnodulating strain 3233 did have five or six high molecular weight plasmids. Strain 620, on the other hand, harbored two large plasmids of 75.8 and 46.8 Kb, respectively. The possible involvement of these plasmids in nodulation and symbiotic nitrogen fixation could not be investigated because of the inavailability of an effective

technique for plasmid isolation from rhizobia. Plasmid transfer between the strains could not be demonstrated most possibly because of the same reason.

6. The inoculum size was the most effective variable in determining the outcome of symbiosis. Although the strains could nodulate the plants at low inoculum levels, nodulation and plant shoot weight increased linearly with increasing inoculum size.

7. With strains 620 and 385, symbiotic effectiveness was highest in soils adjusted to pH 8.0 and pH 9.0, respectively. The strains were more active at a low phosphate concentration of 1.44 mM. With regard to calcium requirement, the inclusion of this mineral at high concentrations was shown to be beneficial only for strain 620.

8. When compared to the agar tubes, Leonard's Jar assemblies were more laborious experimental setups for culturing chickpea, but were more useful in the assessment of nodulation.

9. The statistical analysis of the data obtained from strains 620 and 385 suggested that the nodule number is the most reliable index as it better correlated with the parameters tested. This suggestion was based on the observation that the nodules formed by both strains were healthy; i.e. actively fixing nitrogen. Otherwise, the number of root nodules formed was of not much significance.

10. It is necessary to test the association between bacterium and plant host in a natural and more complex situation. The effectiveness of mixed and single strain inocula is to be evaluated in the field environment so as to further identify the strains for inoculant production.

REFERENCES

Abaidoo, R.C., George, T., Bohloo, B.B., and Singleton, P.W. 1990. "Influence of elevation and applied nitrogen on rhizosphere colonization and competition for nodule occupancy by different rhizobial strains on field-grown soybean and common bean" Can.J.Microbiol., Vol. 36, pp. 92-96.

Afifi, N.M., Moharram, A.A., Hamdi, Y.A., 1969. "Sensitivity of *Rhizobium* Species to Certain Fungicides", Arch. Microbiol., Vol. 66, pp. 121-128.

Alexander, M., 1977. "Ecology of Nitrogen-Fixing Organisms", In Biological Nitrogen Fixation in Farming Systems in Tropics, ed. Ayanaba, A., Dart, P.J. Willey, pp. 94-114, London.

Ali, A.M., Hamdi, Y.A., Ahmad, K.A., Abdel-Wahap, S.M., 1980. "Effectiveness of Nitrogen Fixation in Phage Resistant and Lysogenic *R. trifolii* Strains", Egypt J. Microbiol., Vol. 15, pp. 121-128.

Almendras, A.S., Bottomley, P.J., 1985. "Effects of Phosphorous and Lime upon Nodulation by Indigenous Serogroups of *R. trifolii*", In Nitrogen Fixation Research Progress, Ed. Evans, H.J., Bottomley, P.J., Newton, P.E., Dordrecht, pp. 395, Holland: Nijhoff.

Amarger, N., and Lobreau, J.P., 1982. " Quantitative study of nodulation competitiveness in *Rhizobium* strains", Appl. Environ. Microbiol., Vol. 44, pp. 583-588.

Ames, P., and Bergman, K., 1981. " Competitive advantage provided by bacterial motility in the formation of nodules by *R.meliloti*", J.Bacteriol., Vol. 148, pp. 728-729.

Appelbaum, E., 1990. " The Rhizobium/Bradyrhizobium-Legume symbiosis" In Molecular Biology of Symbiotic Nitrogen Fixation, Gresshoff, P.M. (Ed.), CRC Press, pp. 131, Boca Raton, FL.

Ayanaba, A., Asanuma, S., Munns, D.N., 1983. "An Agar Plate Method for Rapid Screening of *Rhizobium* for tolerance to Acid-Aluminum Stress", Soil Sci. Soc. Am. J., Vol. 47, pp. 256-258.

Banfalvi, Z., Sakanyan, V., Konez , C., Kiss, A., Dusha, I., Kondorosi, A., 1981. "Location of Nodulation and Nitrogen Fixation Genes on a High Molecular Weight Plasmid in *R. meliloti*", Mol. Gen. Genet., Vol. 184, pp. 318-325.

Barea, J.M., Azcon-Aguilar, C., 1983. "*Mycorrhizas* and Their Significance in Nodulating Nitrogen Fixing Plants", Adv. Agron., Vol. 36, pp. 1-54.

Bassam, B.J., Djordjevic, M.A., Redmond, J.W., Batley, M., and Rolfe, B.G., 1988. "Identification of a *nodD*-Dependent Locus in the *Rhizobium* Strain NGR234 Activated by Phenolic Factors Secreted by Soybeans and Other Legumes", Mol. Plant-Microbe Interact., Vol. 1, pp. 161-168.

Bauer, W.D., 1981. "The Infection of Legumes by rhizobia", Ann. Rev. PlantPhysiol., Vol. 32, pp. 407-449.

Beattie, G.A., Clayton, M.K., and Handesman, J. 1989. " Quantitative comparison of the laboratory and field competitiveness of *R.leguminosarum* biovar *phaseoli*", Appl. Environ. Microbiol., Vol. 55, No.11, pp. 2755-2761.

Beck, D.P., Munns, D.N., 1984. "Phosphate Nutrition of *Rhizobium* sp.", Appl. Environ. Microbiol., Vol. 47, pp. 478-482.

Bender, G.L., Goydych, W., Rolfe, B.G., and Nayudu, M., 1987. "The Role of *Rhizobium* Conserved and Host Specific Nodulation Genes in the Infection of the Non-Legume *Parasponia ondersonii*", Mol. Gen. Genet., Vol. 210, pp. 299-306.

Beringer, J.E., Brewin, N.J., Johnston, A.W.B., 1980. "The Genetic Analysis of *Rhizobium* in Relation to Symbiotic Nitrogen Fixation", Heredity, Vol. 45, pp. 161-186.

Beringer, J.E., 1973. "Genetic Studies With *R. leguminosarum*", Ph.D. Thesis, University of East Anglia.

Beynon, J.L., Beringer, J.E., and Johnston, A.W.B., 1980. "Plasmids and host range in *R.leguminosarum* and *R.phaseoli*", J.Gen. Microbiol., Vol. 120, pp. 421-429.

Boonkerd, N., Weber, D.F., and Bezdicek, D.F., 1978. "Influence of *R.japonicum* strains and inoculation methods on soybean grown in Rhizobia-populated soil", Agron. J., Vol. 70, pp. 547-549.

Boonkerd, N., Weaver, R.W., 1982. "Survival of Cowpea *Rhizobia* in Soil as Affected by Soil Temperature and Moisture", Appl. Environ. Microbiol., Vol. 43, pp. 585-589.

Bowen, G.D., Rovira, A.D., 1976. "Microbial colonization of Plant Roots", Ann. Rev. Phytopathol., Vol. 14, pp. 121-144.

Breedveld, M.W., Cremers, H.C.J., Batley, M., Zenhder, A.J.B., 1993. " Polysaccharide synthesis in relation to nodulation behavior of *R.leguminosarum*", J.Bacteriology, Vol. 175(3), pp. 750-757.

Bremner, J.M., 1965. "Total Nitrogen", In Methods of Soil Analysis, (C.A. Black, editor). Amer. Soc. Agronomy, Madison, Part 2, pp. 1149-1178, Wisc.

Brewin, N.J., Beringer, J.E., Buchanan-Wollaston, A.V., Johnston, A.W.B., and Hirsch, P.R., 1980a. " Transfer of symbiotic genes with bacteriocinogenic plasmids in *R.leguminosarum*", J.Gen. Microbiol., Vol. 116, pp. 261-270.

Brewin, N.J., Beringer, J.E., Johnston, A.W. B., 1980b. "Plasmid-Mediated Transfer of Host-Specificity Between Two Strains of *R. leguminosarum*", J. Gen. Microbiol., Vol. 120, pp. 413-420.

Bromfield, E.S.P., Jones, D.G., 1980. "Studies on Acid Tolerance on *R. trifolii* in Culture and Soil", J.Appl. Bacteriol., Vol. 48, pp. 259-264.

Bromfield, E.S.P., Ayanaba, A., 1980. "The Efficiency of Soybean Inoculation on Acid in Tropical Africa", Plant Soil, vol. 54, pp. 95-106.

Broughton, W.J., 1978. "Control of Specificity in Legume-*Rhizobium* Associations", J. Appl. Microbiol., Vol. 45, pp. 165-194.

Bushby, H.V.A., 1982. "In Nitrogen Fixation", Ecology, *Rhizobium* ed. W.J. Broughton, Oxford Univ. Press., Vol. 2, pp. 35-75, Oxford.

Caetano-Anolle's, G., Wall, L.G., de Michaeli, A.T., Macchi, E.M., Favelukes, G., 1985. "Attachment of Nonmotile and Nonchemotactic Mutants of *R. meliloti* to Alfalfa Roots", In Nitrogen Fixation Research Progress ed. Evans, H.J., Bottomley, P.J., pp. 257.

Caetano-Anolle's, G., and Gresshoff, Peter M., 1991. "Plant Genetic Control of Nodulation", Ann. Rev. Microbiol., Vol. 45, pp. 345-382.

Callon, N.W., Manthre, D.E. and Miller, J.B. 1990. " Bio-priming seed treatment for biological control of *Phythium ultimum* preemergance damping-off in sweet corn", Plant Disease, Vol. 74, pp. 368-372.

Casse, F., Boucher, C., 1979. "Identification and Characterization of Large Plasmids in *R. meliloti* Using Agarose Gel Electrophoresis", J. Gen. Microbiol., Vol. 113 , pp. 229-242.

Chakravorty, A.K., Zurkowski, W., Shine, J., Rolfe, B.G., 1982. "Symbiotic Nitrogen Fixation: Molecular Cloning of *Rhizobium* Genes Involved in Exopolysaccharide Synthesis", J. Mol. Appl. Genet., Vol. 1, pp. 585-596.

Chatel, D.L., Parker, C.A., 1973. "Survival of Field-Grown *Rhizobia* Over the Dry Summer Period in Western Australia", Soil Biol. Biochem., Vol. 5, pp. 415-423.

Chen, H., Batley, M., Redmond, J.W., Rolfe, B.G., 1985. "Alteration of the Effective Nodulation Properties of a Fast Growing Broad Host Range *Rhizobium* Due to Changes in Exopolysaccharide Synthesis", J. Plant Physiol., Vol. 120, pp. 331-349.

Copper, J.E., 1982. "Acid Production, Acid Tolerance and Growth Rate of Lotus *Rhizobia* in Laboratory Media", Soil Biol. Biochem., Vol. 14, pp. 127-132.

Curley, R.C., Burton, C., 1975. "Compatability of *R. japonicum* With Chemical Seed Protectants", Agron. J., Vol. 67, pp. 807-808.

Çakmakçi, M.L. 1977. "Bazi bakteri türlerinin saptanmasinda gaz kromatografisi yönteminin uygulanma olanaklari üzerinde arastirmalar" A.Ü. Ziraat Fakültesi, Ziraat Mikrobiyolojisi Kürsüsü, Doktora Tezi,. 123 s.

Dahiya, J.S., and Khurana, A.L., 1981., "Chilum Jar a technique for screening of Rhizobia under summer conditions", Plant Soil, Vol. 63, pp. 299-302.

Damirgi, S.M., Frederick, L.R., Anderson, I.C., 1967. "Serogroups of *R. japonicum* in Soybean Nodules as Affected by Soil Types", Agron. J., Vol. 59, pp. 10-12.

Danso, S.K.A., Keya, S.O., Alexander, M., 1975. "Protozoa and Decline of *Rhizobium* Populations Added to Soil", Can. J. Microbiol., Vol. 21, pp. 884-895.

Dart, P.J., 1977. "Infection and development of leguminous nodules. In A treatise on dinitrogen fixation", R.V.F., Hardy and W.S., Silver (Eds.) Sec. III, Biology, John Wiley and Sons.

Demont, N., Debelle, F., Aurelle, H., Denarie, J., and Prome, J.C., 1993. " Role of the *R.meliloti nod* F and *nod* E genes in the biosynthesis of lipo-oligosaccharidic nodulation factors", J.Biol. Chem., Vol. 268(27), pp. 20134-20142.

Denarie, J., Debelle, F., and Rosenberg, C., 1992. " Signaling and host range variation in nodulation " Ann. Rev. Microbiol., Vol. 46, pp. 497-531.

Detroy, R.W., Witz, D.F., Parejko, R.A., and Wilson, P.W., 1968. In Stanley L. Streicher, Elizabeth G. Gurney, and Raymond C. Valantine, 1972. "The Nitrogen Fixation Genes", Nature, Vol. 239, pp. 495-499.

Djordjevic, Michael A., Gabriel, Dean W., and Rolfe, Barry G., 1987. "*Rhizobium*-The Refined Parasite of Legumes"Ann. Rev. Phytopathol. Vol. 25, pp. 145-168.

Djordjevic, M.A., Zurkowski, W., Shine, J., Rolfe, B.G., 1983. "*Sym* Plasmid Transfer to Symbiotic Mutants of *R. trifolii*, *R. leguminosarum* and *R. meliloti*", J. Bacteriol., Vol. 156, pp. 1035-1045.

Djordjevic, M.A., Schofield, P.R., and Rolfe, B.G., 1985. "Tn5 Mutagenesis of *R. trifolii* Host-Specific Nodulation Genes Result in Mutants With Altered Host-Range Ability", Mol. Gen. Genet., Vol. 200, pp. 463-471.

Djordjevic, M.A., and Weinman, J.J., 1991. "Factors Determining Host Recognition in the Clover-*Rhizobium* Symbiosis", Aust. J. Plant. Physiol., Vol. 18, pp. 543-557.

Doura, C.E., Xenoulis, A.C., Paradellis, T., 1984. "Salinity Tolerance of *Rhizobium meliloti* Strain Isolated From Salt Affected Soils", Folia Microbiol. Praque, Vol. 29, pp. 316-324.

Dowling, D.N., and Broughton, W.J., 1986. "Competition for Nodulation of Legumes", Ann. Rev. Microbiol., Vol. 40, pp. 131-157.

Dudley, M.E., Jacobs, T.W., and Long, S.R., 1987. "Microscopic Studies of Cell Divisions Induced in Alfalfa Roots by *R. meliloti*", Planta, Vol. 171, pp. 289-301.

Dughri, M.H., Bottomley, P.J., 1983. "Effect of Acidity and the Composition of an Indigenous Soil Population of *R.trifolii* Found in Nodules of *Trifolium substerraneum*", Appl. Environ. Microbiol., Vol. 46, pp. 1207-1213.

Dughri, M.H., Bottomley, P.J., 1984. "Soil Acidity and the Composition of an Indigenous Population of *R. trifolii* in Nodules of Different Cultivars of *Trifolium subterraneam*", Soil Biol. Biochem., Vol. 16, pp. 405-411.

Egelhoff, T.T., Fisher, R.F., Jacobs, T.W., Mulligan, J.T., Long, S.R., 1985. "Nucleotide Sequence of *R. meliloti* 1021 Nodulation Genes: *nodD* is Read Divergently From *nodABC* DNA", DNA, Vol. 4, pp. 241-248.

Emerich, D.W., and Wall, J.D., 1985. " Nitrogen fixation. In: Comprehensive Biotechnology", Moo-Young, M. (Ed.) Pergamon Press, Vol. 4, pp. 73-107, New York.

Evans, L.S., Lewin, K.F., Vella, F.A., 1980. "Effect of Nutrient Medium pH on Symbiotic Nitrogen Fixation by *R. leguminosarum* and *Pisum sativum*", Plant Soil, Vol. 56, pp. 71-80.

Faizah, A.W., Broughton, W.J., John, C.K., 1980. "*Rhizobia* in Tropical Legumes XI. Survival in the Seed Environment", Soil Biol. Biochem., Vol. 12, pp. 219-227.

Finan, T.M., Hirsch, A.M., Leigh, J.A., Johansen, E., Kuldau, G.,M.A., 1985. "Symbiotic Mutants of *R. meliloti* That Uncouple Plant From Bacterial Differentiation", Cell, Vol. 40, pp. 869-887.

Finan, T.M., Kunkel, B., de Vos, G.F., Singer, E.R., 1986. "Second Symbiotic Megaplasmid in *R. meliloti* Carrying Exopolysaccharide and Thiamine Synthesis Genes", J. Bacteriol., Vol. 167, pp. 66-72.

Firmin, J.L., Wilson, K.E., Rossen, L., and Johnston, A.W.B., 1986. "Flavonoid Activation of Nodulation Genes in *Rhizobium* Reversed by Other Compounds Present in Plant", Nature, Vol. 324, pp. 90-92.

Franco, A.A., Munns, D.N., 1982. "Acidity and Aluminium Restraints on Nodulation, Nitrogen Fixation and Growth of *Phaseolus vulgaris* in Solution Culture", Soil Sci. Soc. Am. J., Vol. 46, pp. 296.

Gabriel, D.W., 1986. "Specificity and Gene Function in Plant-Pathogen Interactions", Am. Soc. Microbiol. News., Vol. 52, pp. 19-25.

Gegenhermer, P., 1990. "Preparation of Extracts From Plants", Methods in Enzymology, Vol. 182, pp. 174-190.

Gibson, A.H., Nutman, P.S., 1960. "Studies on the Physiology of Nodule Formation", Ann. Bot. London, Vol. 24, pp. 420-433.

Gillberg, B.O., 1971. "On the Effect of Some Pesticides on *Rhizobium* and Isolation of Pesticide Resistant Mutant", Arch. Microbiol., Vol. 75, pp. 203-208.

Göktan, D. 1974. "Türkiye'nin çesitli yerlerinden izole edilen *R.phaseoli* suslarinin etkinlik durumlarinin saptanmasi" H.Ü. Tip Fakültesi Mikrobiyoloji Enstitüsü, Doktora Tezi, 67 s.

Göktan, D. ve Madanoglu, K. 1975. " Nodozite bakteri kültürü ile asilanmanin tarla sartlarinda yonca mahsül verimine ve azot kapsamina etkisi " Top. ve Gübre Aras. Enst. Genel Yayin No: 61, Rapor Seri No: 2, 97 s.

Graham, P.H., Ocampo, G., Ruiz, L.D., Duque, A., 1980. "Survival of *R. phaseoli* in Contact With Chemical Seed Protectants", Agron. J., Vol. 72, pp. 625-627.

Gürbüzer, E. 1973. "*R.meliloti* suslarinin özellikleri ve etkinlik derecelerinin saptanmasi" H.Ü.Tip Fakültesi Mikrobiyoloji Enst., Doktora Tezi, 62 s.

Gürgün, V. 1978. "Bazi nohut Rhizobium suslarinin kültürel karakterleri ile azot tespit ve rekabet etme güçlerinin saptanmasi üzerinde arastirmalar" A. Ü. Ziraat Fakültesi Ziraat Mikrobiyolojisi Kürsüsü, Doktora tezi, 97 s.

Gürgün, V. 1981. " Soya fasülyesi yetistiriciliginde kök nodül bakterilerinin önemi ve biyolojik gübre üretim teknigi " Türkiye I. Soya Kongresi, Adana (Teblig).

Gürgün, V. 1983. " The state of knowledge on symbiotic nitrogen fixation (Rhizobium) in Turkey" FAO Report, pp. 34, Ankara.

Halverson, J.L., Stacey, G., 1986. "Signal Exchange in Plant-Microbe Interactions", Microbial Rev. Vol. 50, pp. 193-225.

Ham, G.E., Caldwell, V.B., Johnson, H.W., 1971. "Evaluation of *R. japonicum* Inoculants in Soil Containing Neutralized Populations of *Rhizobia*", Agron. J., Vol. 63, pp. 301-303.

Handelsman, J., Ugalde, R.A., and Brill, W.J., 1984. "*R.meliloti* competitiveness and the alfalfa aglutinin", J.Bacteriol., Vol. 157, pp. 703-707.

Handelsman,J., Brill, W.J., 1985. "*Erwinia herbicola* Isolates From Alfalfa Plants May Play a Role in Nodulation of Alfalfa by *R. meliloti*", Appl. Environ. Microbiol., Vol. 49, pp. 818-821.

Hardarson, G., Jones, D.G., 1979. "Effect of Temperature on Competition Amongst Strains of *R. trifolii* for Nodulation of Two White Clover Varieties", Ann. Appl. Biol., Vol. 92, pp. 229-236.

Hardy, K.G., 1975. "Colicinogeny and related phenomena", Bacteriological Reviews, Vol. 39, pp. 464-515.

Hardy, R.W.F., Burns, R.C., and Parshall, G.W., 1971. In Stanley, L. Streicher., Elizabeth, G., Gumey and Raymond, C. Valantine., 1972. "The Nitrogen Fixation Genes", Nature, Vol. 239, pp. 495-499.

Hartel, P.G., Alexander, M., 1984. "Temperature and Desiccation Tolerance of Cowpea *Vigna unguiculata Rhizobia*", Can. J.Mirobiol., Vol. 30, pp. 820-823.

Hartel, P.G., Alexander, M., 1983. "Growth and Survival of Cowpea *Rhizobia* in Acid, Aluminum-Rich Soils", Soil Sci. Soc. Am. J., Vol. 47, pp. 502-506.

Herridge, D.F., Roughly, R.J., Brockwell, J., 1984. "Effect of *Rhizobia* and Soil Nitrate on the Establishment and Functioning of the Soybean Symbiosis in the Field", Aust. J. Agric. Res., Vol. 35, pp. 149-161.

Hirsch, P.R., Van Montagu, M., Johnston, A.W.B., Brewin, N.J., and Schell, J., 1980. " Physical identification of bacteriocinogenic, nodulation and other plasmids in strains of *R.leguminosarum*", J.Gen. Microbiol., Vol. 87, pp. 403-412.

Hirsch, P.R., 1978. "Plasmid-Determined Bacteriocin Production by *R. leguminosarum*", J. Gen. Microbiol., Vol. 113, pp. 219-228.

Hodgsen, A.L., Stacey, G., 1986. "Potential for *Rhizobium* Improvement" Crit. Rev. Biotech., Vol. 4, pp. 1-74.

Hohenberg, J.S., Munns, D.N., 1984. "Effect of Soil Acidity Factors on Nodulation and Growth of *Vigna unguiculata* in Solution Culture", Agron. J., Vol. 76, pp. 477-481.

Horvath, B., Bachem, C.W.B., Schell, J., and Kondorosi, A., 1987. "Host Specific Regulation of Nodulation Genes in *Rhizobium* is Mediated by a Plant Signal Interacting With the *nodD* Gene Product", E.M.B.O J., Vol. 6, pp. 841-848.

Hubac, C., Ferran, J., Guerrier, D., Tremolieres, A., Kondorosi, A., 1993. " Luteolin absorption in *R.meliloti* wild-type and mutant strains", J. Gen. Microbiol., Vol. 139(7), pp. 1571-1578.

Hynes, M.F., Simon, R., Muller, P., Niehaus, K., Labes, M., Puhler, A., 1986. "The Two Megaplasmids of *R. meliloti* Are Involved in the Effective Nodulation of Alfalfa", Mol. Gen. Genet., Vol. 202, pp. 356-362.

Innes,R.W., Kuempel, P.L., Palajinski, J., Canter-Cremers, H., Rolfe, B.G., and Djordjevic, M.A., 1985. "Plant Factors Induce Expression of Nodulation and Host-Range Genes in *R. trifolii*", Mol. Gen. Genet., Vol. 201, pp. 426-432.

Innes, R.W., Hirose, M.A., and Kuempel, P.L., 1988. "Induction of Nitrogen-Fixing Nodules on Clover Roots Requires only 32 Kb Pairs From the *R. trifolii* Symbiosis Plasmid", Journal of Bacteriology, Vol. 170, pp.3793-3802.

Johnston, A.W.B., 1990. Biological Nitrogen Fixation. In "A Revolution in Biotechnology", J.L. Marx (Eds.) Cambridge University Press, Cambridge, pp. 103-118, New York.

Jones, D.G., Burrow, A.C., 1969. "Acid Production and Symbiotic Effectiveness in *R. trifolii*", Soil Biol. Biochem., Vol. 1, pp. 57-61.

Josey, D.P., Beynon, J.L., Johnston, A.W. and Beringer, J.E., 1979. " Strain identification in *Rhizobium* using intrinsic antibiotic resistance", J.App. Bacteriol., Vol. 46, pp. 343-350.

Joshi, M.M., Hillebrenner, S.N., Goss, G.R., 1981. "Effect of Water Potential on the Survival of *R. japonicum* in Two Soils", Phytopathology, Vol. 71, pp. 229.

Kao, T.C., Wang, C.C., 1981. "Effect of Herbicides on Growth of *Rhizobia* and Development of Root Nodules. 4. The Nodulation Efficiency of Herbicide Treated *Rhizobia*", J. Agric. China New Ser., Vol. 114, pp. 61-71.

Keya, S.O., Alexander, M., 1975."Regulation of Parasitism by Host Density: The Bdellovibrio-*Rhizobium* Interrelationship", Soil Biol. Biochem., Vol. 7, pp. 231-237.

Kiss, G.B., and Kalman, Z., 1982. " Transformation of *R.meliloti* 41 with plasmid DNA ", J. Bacteriology, Vol. 150, No. 2, pp. 465-470.

Kitis, G. 1976. " Mercimek kök nodül bakterilerinin izolasyonun, karakteristikleri ve nitrojen tesbit etme güçleri üzerinde arastirmalar" A.Ü. Ziraat Fakültesi, Ziraat Mikrobiyolojisi Kürsüsü Ihtisas Tezi, 70 s.

Kondorosi, A., Kondorosi, E., John, M., Schmidt, J., and Schell, J., 1991. " The role of nodulation genes in bacterium-plant communication", In Genetic Engineering, Setlow, J.K. (Ed.) Plenum Press, Vol. 13, pp. 115, New York.

Kondorosi, E., Banfalvi, Z., and Kondorosi, A., 1984. "Physical and Genetical Analysis of a Symbiotic Region of *R. meliloti:* Identification of Nodulation Genes", Mol. Gen. Genet., Vol. 193, pp. 445-452.

Kosslak, R.M., Bohlool, B.B., 1985. "Influence of Environmental Factors on Interstrain Competition in *R. japonicum*", Appl. Environ. Microbiol., Vol. 49, pp. 1128-1133.

Krischman, H.B., and Pueppke, S.G., 1993. " Flavanoid inducers of nodulation genes stimulate *R.fredii* USDA 257 to export proteins into the environment", Mol. Plant-Microbe Interac., Vol. 6(1), pp. 107-113.

Kucey, R.M.N., Paul, E.A., 1982. "Carbon Flow, Photosynthesis and Nitrogen Fixation in Mycorrhizal and Nodulated Faba Beans, *Vicia faba*", Soil Biol. Biochem., Vol. 14, pp. 407-412.

Lakshminarayana, K., Narula, N., and Tauro, P., 1988. " A simple technique to obtain nodulation of chickpea and some large-seeded legumes in agar tubes". Tropic. Aqric., (Trinidad), Vol. 65, pp. 121-124.

Lamb, J.W., Hennecke, H., 1986. "In *B. japonicum* the Common Nodulation Genes, *nodABC*, are Linked to *nifA* and *fixA*", Mol. Gen. Genet., Vol.202, pp. 512-517.

Leigh, J.A., Signer, E.R., Walker, G.C., 1985. "Exopolysaccharide-Deficient Mutants of *R. meliloti* That Form Ineffective Nodules", Proc. Natl. Acad. Sci. USA, Vol. 82, pp. 6231-6235.

Ligon, J.M., 1990. Molecular Genetics of Nitrogen Fixation in Plant-Bacteria Symbioses. In "Biotechnology of Plant-Microbe Interaction", J.P. Nakes and C. Hagedorn (Eds), Mac Gro. Hill Pulb. Company, pp. 145-178, New York.

Long, S.R., 1984. "Genetics of *Rhizobium* Nodulation", In Plant-Microbe Interactions, ed. T. Kosuge, E.W. Nester, Macmillan, Vol. 1, pp. 265-306, New York.

Long, S.R., 1989. "*Rhizobium*-Legume Nodulation: Life Together in the Underground", Cell, Vol. 56, pp. 203-214.

Lotz, W., and Mayer, F., 1972. " Isolation and characterization of a bacteriophage tail-like bacteriocin from a strain of Rhizobium", J.Virology, Vol. 9, pp. 160-173.

Lowendorf, H.S., Alexander, M., 1983. "Identification of *R. phaseoli* Strains That Are Tolerant or Sensitive to Soil Acidity", Soil Sci. Soc. Am. J., Vol. 47, pp. 935-938.

Lowry, O.H., Rosebrough, N.G., Farr, A.L., and Randall, R.J., 1951. " Protein measurement with the folin-phenol reagent". J. Biol. Chem., Vol. 193, pp. 265-275.

Magu, S.P., Bhowmik, S., 1984. "Effect of 2-methyl-4-chloro-phenoxybutyric acid and Disyston Legume and *Rhizobium* Symbiosis and Rhizosphere Microflora", Zentralbl. Mikrobiol., Vol. 139, pp. 633-641.

Mahler, R.L., Wollum, A.G., 1981. "The Influence of Soil Water Potential and Soil Texture on the Survival of *R. japonicum* and *R. leguminosarum* Isolates in the Soil", Soil Sci. Soc. Am. J., Vol. 45, pp. 761-766.

Makawi, A.A., Abdel-Ghaffar, A.S., 1970. "The Effect of Some Pesticides on the Growth of Root-Nodule Bacteria", UAR J. Mirobiol., Vol. 5, pp. 1-2, 109-117.

Maniatis, T., Fritsch, E.F., and Sambrook, J. 1982. " Molecular cloning", A Laboratory Manual Cold Spring Harbor, pp. 90, New York.

Marschner, H., 1986. " Introduction, definition and classification of mineral nutrients", Mineral Nutrition in higher Plants; Academic press, pp.3, San Diego

Martensson, A.M. 1990. "Competitiveness of inoculant strains of *R.leguminosarum* bv. *trifolii* in red clover using repeated inoculation and increased inoculum levels", Can. J. Microbiol., Vol. 36, pp. 136-139.

May, S.N., Bohlool, B.B., 1983. "Competition Among *R. leguminosarum* Strains for Nodulation of Lentils (*Lens esculenta*)", Appl. Environ. Microbiol. Vol. 45, pp. 960-965.

McNeil, D.C., 1982. "Variations in Ability of *R. japonicum* Strains to Nodulate Soybean and Maintain Fixation in the Presence of Nitrate", Appl. Environ. Microbiol., Vol. 44, pp. 647-652.

Milagros, L.B., Felix, D.D., Cecillia, M.J., and Donald, A.P. 1993. " Isolation of *R.meliloti nod* gene inducers from alfalfa rhizosphere soil", Appl. Environ. Microbiol., Vol. 59(2), pp. 636-639.

Mortenson, L.E., 1968. In Stanley, L. Streicher., Elizabeth, G., Gumey and Raymond, C. Valentine., 1972. "The Nitrogen Fixation Genes", Nature, Vol. 239, pp. 495-499.

Nap, J.P., and Bisseling, T., 1990. " Developmental biology of a plant-prokaryote symbiosis: The legume root nodule ", Science, Vol. 250, pp. 948-954.

O'Connell, M.P., Hynes, M.F., Puhler, A., 1987. "Incompatibility Between a *Rhizobium* Sym Plasmid and a Ri Plasmid of *Agrobacterium*", Plasmid, Vol. 18, pp. 156-163.

O'Hara, G.W., Boonkerd, N., and Dilworth, M.J., 1988a. "Mineral constraints to nitrogen fixation", Plant soil, Vol. 108, pp. 93.

O'Hara, G.W., Dilworth, M.J., Bookard, and Parkpian, P., 1988b. "Iron deficiency specifically limits nodule development in peanut inoculated with *Bradyrhizobium* spp." New Phytol., Vol. 108, pp.51.

Osa-Afiana, L.O., Alexander, M., 1982. "Differences Among Cowpea *Rhizobia* in Tolerance to High Temperature and Desiccation in Soil", Appl. Environ. Microbiol., Vol. 43, pp. 435-439.

Pankhurst, C.E., Layzell, D.B., 1984. "The Effect of Bacterial Strain and Temperature Changes on the Nitrogenase Activity of *Lotus pedinculatus* Cultivar Maku Root Nodules", Physiol. Plant, Vol. 62, pp. 404-409.

Parker, C.A., Grove, P.L., 1970. "Bdellovibrio Bacteriovirus Parasitizing *Rhizobia* in Western Australia", J. Appl. Bacteriol., Vol. 33, pp. 253-255.

Pena-Cabriales, J.J., Alexander, M., 1983. "Growth of *Rhizobium* in Soil Amended With Organic Matter", Soil Sci. Soc. Am. J., Vol. 47, pp. 241-245.

Pimental, D., 1975. In "Molecular Biology of Nitrogen Fixation", Science, Vol.187, pp.919-924.

Pretorius-Guth, I.G., Pühler, A., and Simon, R., 1990. " Conjugal transfer of megaplasmid between *Rhizobium meliloti* strains in alfalfa nodules.", Appl. Environ. Microbiol., Vol. 56, pp. 2354.

Rai, R., Singh, S. N., and Prasad, V., 1982. "Effect of presumed amend pyrite on symbiotic N_2-fixation, active iron contents of nodules, grain yield and quality of chickpea (*Cicer arientinum* L.) genotypes in calcareous soil ", J.Plant Nutr., Vol. 5, pp. 905.

Ramirez, C., Alexander, M., 1980. "Evidence Suggesting Protozoan Predation on *Rhizobium* Associated with Germinating the Seed in the Rhizosphere of Beans", Appl. Environ. Microbiol., Vol. 40, pp. 492-499.

Richardson, A.E., Simpson, R.J., Djordjevic, M.A., and Rolfe, B.G., 1988. " Expression of nodulation genes in *R.leguminosarum* biovar *trifolii* is affected by low pH and by Ca and Al ions". Environ. Microbiol., Vol. 54, pp. 2541.

Rosenberg, C., Boistard, P., Denarie, J., Casse-Delbert, F.L.,1981. "Genes Controlling Early and Late Functions in Symbiosis are Located on a Megaplasmid in *R. meliloti*", Mol. Gen. Genet., Vol. 184, pp. 326-333.

Roslycky, E.B. 1967. "Bacteriocin production in the Rhizobia bacteria", Can. J.Microbiol., Vol. 13, pp. 431-532.

Rossen, L., Johnston, A.W.B., and Downie, J.A., 1984. "DNA Sequence of the *R. leguminosarum* Nodulation Genes *nodAB* and *C* Required for Root Hair Curling", Nucleic Acids Res., Vol. 12, pp. 9497-9508.

Rossen, L., Shearman, C.A., Johnston, A.W.B., Downie, J.A., 1985. "The nodD Gene of *R. leguminosarum* is Autoregulatory and in the Presence of Plant Exudate Induces the Expression of *nodABC* Genes", EMBO J., Vol. 4, pp. 3369-3373.

Roughley, R.J., Bromfield, E.S.P., Pulver, E.L., Day, J.M., 1980. "Competition Between Species of *Rhizobium* for Nodulation of *Glycine max.*", Soil Biol. Biochem., Vol. 12, pp. 467-470.

Russell, P.E., Jones, D.G., 1975." Variation in the Selection of *R. trifolii* by Varieties of Red and White Clover", Soil Biol. Biochem., Vol. 7, pp. 15-18.

Russell, P., Shell, G.M., Nelson, K.K., Halverson, L.J., Sirotkin, K.M., Stacey, G., 1985. "Isolation and Characterization of the DNA Region Encoding Nodulation Functions in *B. japonicum*", J. Bacteriol., Vol. 164, pp. 1301-1308.

Schiaman, H.R.M., 1992. " Regulation of nodulation gene expression in *R. leguminosarum* biovar *viceae* ", Ph.D Thesis. Leiden University, Leiden, the Netherlands.

Schofield, P.R., Ridge, R.W., Rolfe, B.G., Shine, J., and Watson, J.M., 1984. "Host-Specific Nodulation is Encoded on a 14 Kb DNA Fragment in *R.trifolii*", Plant Molecular Biology, Vol. 3, pp. 3-11.

Schwinghamer, E.A. and Belkengren, R.P. 1968. " Inhibition of rhizobia by a strain of *R.trifolii*. Some properties of the antibiotic and of the strain ", Archiv für Mikrobiologie, Vol. 64, pp. 130-145.

Schwinghamer, E.A., 1971. " Antagonism between strains of *R.trifolii* in culture Soil Biol. an Biochem., Vol. 3, pp. 355-363.

Schwinghamer, E.A., 1975. " Properties of some bacteriocins produced by *R.trifolii*", J. General Microbiol., Vol. 91, pp. 403-413.

Schwinghamer, E.A., Pankhurst, C.E., Whitfield, P.R., 1973. " A phage-like bacteriocin of *R.trifolii*", Can. J. Microbiol., Vol. 13, pp. 359-368.

Schwinghamer, E.A., Brockwell, J., 1978. "Competitive Advantage of Bacteriocin and Phage Producing Strains of *R. trifolii* in Mixed Culture", Soil Biol. Biochem., Vol. 10, pp. 383-387.

Schwinghamer, E.A., Reinhardt, D.J., 1975. "Lysogeny in *R.leguminosarum* and *R. trifolii*", Aust. J. Biol. Sci., Vol. 16, pp. 597-605.

Schwinghamer, E.A., 1975. "Properties of Some Bacteriocins Produced by *R. trifolii*", J. Gen. Microbiol., Vol. 91, pp. 401-413.

Scott, K.F., 1986. "Conserved Nodulation Genes From the Non-Legume Symbiont *Bradyrhizobium* sp. (*Parasponia*)", Nucleic Acids Res., Vol. 14, pp. 2905-2919.

Shanmugam, K.T., and Valentine, C.R., 1975. " Molecular biology of nitrogen fixation", Science, Vol. 187, pp. 919-924.

Singleton, P.W., 1983. "A Split Root Growth System for Evaluating the Effect of Salinity on Components of the Soybean *R. japonicum* Symbiosis", Crop Sci., Vol. 23, pp. 259-262.

Somasegaran, P., Hoben, H.J., 1985. In "Methods in Legume-*Rhizobium* Technology", University of Hawaii NifTAL Project and MIRCEN Department of Agronomy and Soil Science.

Somasegaran, P., Hoben, H.J., and Gurgun, V. 1988. " Effects of inoculum rate, Rhizobial strain composition, and nitrogen fixation in chickpea" Agronomy J., Vol. 80, No:1, pp. 68-73.

Sousa, C., Folch, J.L., Boloix, P., Megias, M., and Quinto., 1993. " A *Rhizobium tropici* DNA region carrying the amino-terminal half of a *nod* D gene and a *nod*-box-like sequence confers host-range extension", Molec. Microbiol., Vol. 9(6), pp. 1157-1168.

Sprent, J.I., 1986. " Benefits of *Rhizobium* to agriculture", Trends in Biotechnology, Vol. 4, pp. 124-129.

Stacey, G., Paau, A.S., Noel, K.D., Maier, R.J., Silver, L.E., Brill, W.J., 1982. "Mutants of *R.japonicum* Defective in Nodulation", Arch. Microbiol., Vol. 132, pp. 219-224.

Stacey, G., Pocratsky, L.A., Puvanesarajah, V., 1984. "Bacteriophage That Can Distinguish Between Wild Type *R. japonicum* and a Non-Nodulating Mutant", Appl. Environ. Microbiol., Vol. 48, pp. 68-72.

Stanley, L. S., Elizabeth, G., Gurney and Raymond, C. Valantine.,1972. " The Nitrogen Fixation Genes ", Nature, Vol. 239, pp. 495-500.

Stolp, H., 1973. "The Bdellovibrios: Bacterial Parasites of Bacteria", Ann. Rev. Phytopathol., Vol. 2, pp. 53.

Tan, I. K.P., Broughton, W.J., 1981. "*Rhizobia* in Tropical Legumes. XIII. Biochemical Basis of Acid and Alkali Reactions", Soil Biol. Biochem., Vol. 13, pp. 389-393.

Thornton, F.C., Davey, C.B., 1983. "Response of the Clover-*Rhizobium* Symbiosis to Soil Acidity and *Rhizobium* Strain", Agron. J., Vol. 75, pp. 557-560.

Townshend, J.K., 1989. " Efficacy of oxamyl coated on alfalfa seed with a polymer sticker in *Pratylenchus* and *Meloidogyme* infected soils", J.Nematology, Vol. 21, pp. 242-246.

Triplett, E.W., 1990. " The molecular genetics of nodulation competitiveness in *Rhizobium* and *Bradyrhizobium* ", Mol. Plant-Microbe Interac., Vol. 3, pp. 199.

Triplett, E.W., and Barta, T.M., 1987. "Trifolitixin production and nodulation are necessary for the expression of superior nodulation competitiveness by *R.leguminosarum* bv. *trifolii* strain T24 on clover", Plant Physiol., Vol. 85, pp.335-342.

Ugalde, R.A., Handelsman, J., and Brill, W.J., 1986. "Role of Galactosyltransferase activity in phage sensitivity and nodulation competitiveness of *R.meliloti*", J.Bacteriol., Vol. 166, pp. 148-154.

Ulrich, A., Pühler, A., 1994. " The new class II transposon Tn 163 is plasmid-borne in two unrelated *R.leguminosarum* biovar *viciae* strains ", Mol. Gen. Genet., Vol. 242, pp. 505-516.

Ulgen, H. 1978. " Orta Anadolu kosullarinda Fig bitkisinin urun miktarinda ve azot kapsaminda en fazla artis saglayan nodozite bacteri suslarinin, sera ve tarla sartlarinda secilmesi" Toprak ve Gubre Arastirma Enstitusu Yayinlari, Genel yayin N: 79, Rapor Yayin No: 13.

Van Rensberg, H.J., Strijdom, B.W., 1982. "Competitive Abilities of *R. meliloti* Strains Considered to Have Potential as Inoculants", Appl. Environ. Microbiol., Vol. 44, pp. 98-106.

Vincent, J.M., 1970. A Manual for the Practical Study of Root-Nodule Bacteria. IBP Handbook No : 15. Blackwell Scientific Pube., Oxford.

Vincent, J.M., 1980."Factors Controlling the Legume-*Rhizobium* Symbiosis" In Nitrogen Fixation, ed. W.E. Newton, W.H. Orme-Johnson. Baltimore;Univ, Park Press., Vol. 2, pp. 103-129.

Wacek, T.J., and Brill, W.J., 1976. " Simple rapid assay for screening N_2-fixing ability in soybean", Crop Sci., Vol. 16, pp. 519- 523.

Weber, C.F., Miller, V.L., 1972. "Effect of Soil Temperature on *Rhizobium japonicum* Serogroup Distribution in Soybean Nodules", Agron. J., Vol. 64, pp. 796-798.

Yap, S.F., Lim, S.T., 1983. "Response of *Rhizobium* Strains UMKL20 to Sodium Chloride Stress", Arch. Mirobiol., Vol. 135, pp. 224-228.

Zajac, E., Lorkiewcz, Z., 1983. "Role of O-Acetyl Groups in the Lipopolysaccharide Receptor for *Rhizobium* Phage IP", Gen. Microbiol., Vol. 129, pp. 3171-3176.

APPENDICES

APPENDIX A

CHEMICALS AND SUPPLIERS

Chemicals:	Suppliers:
Agar	Oxoid
Agarose	Sigma
Yeast Extract	Oxoid
Sodium Chloride	Merck
Peptone	Oxoid
Mannitol	Merck
L-tyrosine	Sigma
EDTA	Sigma
Tris-HCl	Merck
SDS	Merck
Potassium Acetate	Merck
Phenol	Merck
Ethanol	Tekel
8-Hydroxy Quinoline	Sigma
K_2HPO_4	Merck
KH_2PO_4	Merck
$CaCl_2.2H_2O$	Merck
$MnSO_4.H_2O$	Merck
$MgSO_4.7H_2O$	Merck
$ZnSO_4.7H_2O$	Merck
$CuSO_4.5H_2O$	Merck
H_3BO_3	Merck
$Na_2MoO_4.2H_2O$	Merck
$CaSO_4$	Merck
Fe(III) Citrate	Merck

NaOH	Merck
HCl	Merck
Streptomycin	Sigma
Spectinomycin	Sigma
Lincomycin	Sigma
Kanamycin	Sigma
Chloramphenical	Sigma
Cloxacillin	Sigma
Gentamicin	Sigma
Nalidixic acid	Sigma
Tetramycin	Sigma
Amikasin	Sigma
Ampicillin	Sigma
Neomycin	Sigma
Hydrogen peroxide	İlka Kimyasal
Tricarboxylic acid	Sigma

APPENDIX B

COMPOSITION AND PREPARATION OF CULTURE MEDIA

1. Yeast Extract Mannitol (YEM) Broth:

Component:	g/lt
K_2HPO_4	0.5
$MgSO_4.7H_2O$	0.2
NaCl	0.1
Mannitol	10.0
Yeast Extract	1.0

pH 6.8-7.0

Sterilization at 121°C for 15 minutes.

2. Tryptone-Yeast Extract (TY) Broth:

Component:	g/lt
Difco Bactone-Tryptone	5
Difco Bacto-Yeast Extract	3
$CaCl_2$	0.77

Sterilization at 121°C for 15 minutes.

3. N-free Jensen Medium:

Component:	mg/lt
K_2HPO_4	360
KH_2PO_4	120
$MgSO_4.7H_2O$	250
$CaSO_4$	250
Fe(III) Citrate	30

Trace elements:

Components:	mg/lt
$MnSO_4.H_2O$	1.00
$ZnSO_4.7H_2O$	0.25
$CuSO_4.5H_2O$	0.25
H_3BO_3	0.50
$Na_2MoO_4.2H_2O$	0.05

Sterilization at 121°C for 1 h with Leonard Jar System.

4. Mueller Hilton Agar

Mueller Hilton broth	21g/lt
Agar powder	15g/lt

Sterilization at 121°C for 20 minutes.

APPENDIX C

COMPOSITION OF BUFFERS

1. TE Buffer (Maniatis, 1982)

50 mM Tris-HCl pH: 8.0
20 mM EDTA pH: 8.0

2. Lysing solution (Casse, 1979)

TE Buffer
%1 SDS
10 N NaOH
final pH: 12.45

3. Tris-Cl buffer (Maniatis, 1982)

2 M Tris-Cl (pH: 7.0)

4. Potassium Acetate (Maniatis, 1982)

Potassium acetate	5 M 60 ml
Glacial acetic acid	11.5 ml
Distilled water	28.5 ml
Total volume:	100 ml

5. TES Buffer (Casse, 1979)

Tris-Cl	0.05 M (pH: 8.0)
EDTA	0.005 M (pH: 8.0)
NaCl	0.05 M

6. TBE Buffer (Maniatis, 1982)

0.089 M Tris-base pH: 8.0-8.3
0.89 M Boric acid
0.002 M EDTA

7. NaCl stock

20.71 g NaCl in 100 ml distilled water.