Improvement of common bean (*Phaseolus vulgaris*) nodulation by selected rhizobial strains from Egyptian soils through genotypic characterization, symbiotic effectiveness and competitiveness under salt stress conditions

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Dedication

To my whole family who have given me much love and support

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List of abbreviations

AMPD	2-amino-2-methyl-1,3 propanediol
APS	ammonium peroxide persulfate
ARA	acetylene reduction assay
ARDRA	amplified ribosomal DNA restriction analysis
bp	base pair
BLAST	basic local alignment search tool
bv	biovar
CBB	commassie brilliant blue
CHAPS	[3-cholamidopropyl-dimethyl amonio]-1-propane sulfonate
α CHCA	α-cyano-4-hydroxycinnamic acid
CTAB	cetyltrimethyl ammonium bromide
DMF	dimethyl formamide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DIG	digoxigenin
dt	dry ton
dNTP's	neuclotide bases
DTT	dithiothreitol
EC	electric conductivity
EDTA	etylenedriamintetra acetic acid
ERIC	repetitive intergenic consensus
EtBr	ethidium bromide
Et	ethanol
FAOSTAT	Food Agricultural Organization
fix	symbiotic nitrogen fixation gene
FW	fresh weight
g	gram
h	hour
ha	hectare
HOMOPIPES	hexamethyleneimine
IEF	isoelectric focusing
IPG	immobiline pH gradient
ITS	internally transcribed spacer
Kb	Kilobase
KD	Kilodalton
LB	Lauria Bertani, a medium for *E. coli*
LSD	least significant difference
M	molarity
mA	milliamper
MALDI	matrix assisted laser adsorption ionization

mbar	millibar
Mb	mega base pair(s)
MD	mega Dalton
MES	morpholinoethanesulphonic acid
mg	milligram
min	minute
MM	minimal media
MS	mass spectrometer
MW	molecular weight
NBT	nitro blue tetrazolium salt
NCBI	National Center for Bioinformatics Institute
nif	nitrogenase structural gene
nm	nanometer
nod	nodulation genes
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
pH	reciprocal of the H^+ concentration
PMF	peptide mass fingerprint
ppm	parts per million
PY	tryptone yeast
rDNA	ribosomal DNA
REP	repetitive extragenic palindromic sequences
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rpm	revolutions per minute
rt	room temperature
SDS	sodiumdodeocyl sulphate
sec	second
Str	streptomycin
Sp	spectinomycin
TAE	tris acetate EDTA
TBE	tris borate EDTA
TCA	trichloroacetic acid
TE	tris EDTA
TEMED	N, N, N, N, tetramethylethylenediamine
TFA	trifluro acetic acid
TIEF	tagged image file formate
TOF	time of flight
tRNA	transfer ribonucleic acid
UPGMA	Unpaired group method using arithmetic averages
UV	ultra violet
V	volt

v/cm	volt per centimeter
v/v	volume by volume
W	watt
Wt	wild type
w/v	weight per volume
x-gluc	5-bromo-4-chloro-3-indoalyl glucuronide
x-phosphate	5-bromo-4-chloro-3-indoalyl phosphate salt

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1 Introduction

1.1 General introduction

Phaseolus vulgaris has its origin in Middle- and South-America. It is a major world crop with almost 20 million tons of seeds produced per year, ranking directly after soybean and peanuts in the world production of grain legumes (FAOSTAT 2001). The area on which common beans are produced is more than 27 million ha, with an average production of around 7 dt per ha. The highest production rates are found in Germany with 26 dt per ha whereas the production rates in African countries are around 7 dt per ha. This means, there is a large potential for increasing the productivity of *Phaseolus vulgaris* in Africa Kratzsch (1999). In Egypt, Common bean is widely consumed as vegetables and also as dry seeds. The annual cultivated area is between 9−18 thousand hectares, yielding 15,000 tons of dry seeds and 150,000 tons of green pods (data from the Central Administration of Agriculture in Egypt CAAE, 1994). Different *Rhizobium* species are able to nodulate and fix nitrogen with the common bean (*Phaseolus vulgaris*), including *Rhizobium leguminosarum* bv. *phaseoli* (Jordan, 1984), *R. tropici* (Martinez et al., 1991), *R. etli* (Segovia et al., 1993), *R. gallicum* and *R. giardinii* (Amarger et al., 1997). The percentage of biological nitrogen fixation of the N assimilation in *Phaseolus vulgaris* is, compared to other legumes, rather low, with 40−50% compared to 75% with faba beans, 70% with peas and up to 95% with lupines (Werner, 1999). Failure of nodulation or lack of response to inoculation in the field has been reported, raising doubts about the benefits of inoculation (Buttery et al., 1987; Graham, 1981). Factors that can limit inoculation benefits could be the promiscuity observed in *Phaseolus vulgaris* (Hernandez et al., 1995; Michiels et al., 1998). Soil conditions are the most important factors restricting nodulation with beans, including root temperature (Weaver and Holt, 1990), salinity (Bordeleau and

Prevost, 1994), acidity (Arons and Graham, 1991), and also the effect of high N−fertilizer concentration used in intensive agriculture (Temperano et al., 1997) or changes in the genome of microsymbionts (Girard et al., 1991). The genetic biodiversity of *Rhizobium* in bean nodules is significantly affected by soil fertilization, especially by ammonia and nitrate (Caballero−Mellado et al., 1999). The complexity of the genome of *Rhizobium etli* is especially large since it is distributed among 7 replicons, one chromosome with 5Mb and 6 plasmids with together 2.2 Mb (Bustos et al., 2002; Sessitsch et al., 2002). From different regions in the world there are many reports as well on genetic uniformity (Wegener et al., 2001) and on large biodiversity (Castro−Sowinski et al., 2002), but little information is available about rhizobial strains nodulating common bean in Egypt. Based on these reports our research was directed to:

1− Isolate new effective strains from Egyptian soils, characterizing their nodulation efficiency on bean cultivars from Egypt and other countries.

2− Studying the genetic diversity of these strains by molecular methods such as, REP−PCR, the amplified ribosomal DNA restriction analysis (ARDRA) of 16S and 23S rDNA, plasmid analysis and 16S rDNA sequencing.

3−Screening to select the strains best adapted to environmental stresses such as salinity, alkalinity, acidity and temperature.

4−Select the most competitive and adapted strains to environmental conditions in Egypt using *Gus* reporter gene.

5− Trials to identify some proteins involved in salt tolerance.

1.2 Taxonomy of *Rhizobium* nodulating *Phaseolus vulgaris*

The early classification of rhizobia was maintained on the ability of infection and nodulation of particular legume species (Somasegaran and Hoben, 1985). Jordan (1982 and 1984) classified rhizobia on the basis of (a) numerical taxonomy, (b) DNA mole percent of guanine and cytosine (c) nucleic acid hybridization (d) cistron similarities (e) serological relationship (f) extra cellular

polysaccharide composition (g) carbohydrate utilization patterns and metabolic capacities (h) antibiotic sensitiveness (i) protein banding patterns and growth rate of bacteria. According to these criteria, rhizobia were classified into two genera*, Rhizobium* and *Bradyrhizobium*. The organism previously designated as *R. leguminosarum*, *Rhizobium trifolii* and *Rhizobium phaseoli* were combined into a single species, *Rhizobium leguminosarium,* with three biovars based mainly on the presence of different symbiotic plasmids coding for distinct nodulation specificity in uniform chromosomal backgrounds.

Two different types of symbiotic plasmid among rhizobial isolates were obtained from bean nodules as reported by Martinez et al. (1985). Symbiotic plasmids of type I strains have multiple copies of *nifH* gene (Quinto et al., 1982); Martinez et al., 1985), a narrow nodulation host range and hybridize with Psi (polysaccharide inhibition) gene (Borthakur et al., 1985). On the other hand, the symbiotic plasmids of type ∏ strains have a single copy of *nifH* gene, nodulate *Leucaena spp*. and do not hybridize with the psi gene (Martinez et al., 1988). Martinez et al. (1991) proposed a new species named *R. tropici* which revealed further subdivision into two similar but distinct subgroups using multilocus enzyme electrophoresis for 65 strains of *R. leguminosarum* bv. *phaseoli* type ∏.

Segovia et al. (1993) reported that *R. leguminosarum* bv. *phaseoli* type I strains could be reclassified on the basis of a sequence analysis of 16S ribosomal RNA, as a new species, named *R. etli*. This taxonomy is based on (a) capacity to establish an effective symbiosis with common bean plants (b) the nitrogenase structural genes (c) organization of the common bean nodulation genes into two separate transcriptional units bearing the *nod*A and *nod*BC (d) presence of the polysaccharide inhibition gene Psi and (e) the 16S ribosomal RNA sequence (Young et al., 1991).

Amarger et al. (1997) proposed two new species of *Rhizobium* that nodulate *Phaseolus vulgaris* in France based on the results of DNA–DNA

hybridization experiments, restriction analysis of the amplified fragments of 16S rDNA and sequence of 16S rDNA. They compared the sequence analysis for two species from France with the sequence analysis for 64 strains from data banks. The phylogenetic tree revealed that each species formed an independent lineage different from the lineages observed from the described species of *Rhizobium*-symbionts of *Phaseolus vulgaris*. The genome sequence for species 1 clustered with the species that was able to nodulate and fix nitrogen with common bean as well as *Rhizobium leguminosarum*, *Rhizobium etli* and *Rhizobium tropici*, and branched with unclassified rhizobial strain OK50, which was isolated from root nodules of *Petrocarpus klemmei* in Japan. Genomic species 2 was distantly related to all other *Rhizobium* species and related taxa, and it was closely related to *Rhizobium galegae* and several *Agrobacterium* species. The proposed names for these two new species were *R. gallicum* and *R. giardinii*, each with two biovars based on the symbiotic and molecular characteristics. *Rhizobium gallicum* bv. *gallicum* nodulating *Phaseolus spp*., *L. leucocephala*, *Macroptillium atropurpureum* and *Onobrychis vicifolii* had only one single copy of the nitrogenase *nifH* gene. *Rhizobium gallicum* bv. *phaseoli* nodulating *Phaseolus spp*., and *Macroptillium atropurpureum,* failed to nodulate *L. leucocephala* had three copies from the *nifH* gene. *Rhizobium giardinii* bv. *giardinii* is able to nodulate the same hosts as *R. gallicum* bv. *gallicum,* failed to hybridize with *nifH* gene. *Rhizobium giardinii* bv. *phaseoli* is weakly efficient in nitrogen fixation with *Phaseolus vulgaris.* They can also nodulate *Macroptillium atropurpureum* after delaying in nodulation for at least one month, and also characterized by three copies of *nifH* gene.

1.3 Differentiation among rhizobial isolates using molecular methods

1.3.1 PCR based on Repetitive Extragenic Palindromic (REP) sequences

Many methods to characterize and identify rhizobial strains are available, but in the last two decades scientists established a new approach to study the diversity of indigenous *Rhizobium* strains using the Polymerase Chain Reaction

(PCR). PCR can be performed rapidly with strains, species or genus specific primers that generate fingerprint characteristics of each strain. DNA primers corresponding to repetitive sequences that present in multiple copies of the genomes of most Gram-negative and Gram-positive bacteria can be used to fingerprint the genomes of rhizobial strains (Versalovic et al. 1991; De Bruijn, 1992). Three families of repetitive sequences have been identified, including repetitive extragenic palindromic (REP), repetitive interagenic consensus (ERIC) and BOX element (Versalovic et al., 1994). The REP-PCR genomic fingerprinting protocols (Rademaker et al.,1998; Versalovic et al., 1994) have been successfully used in a wide variety of Eubacteria for typing strains and studying their diversity. Several investigators (De Bruijn, 1992; Judd et al., 1993; Nick and Lindström, 1994; Selenska-Pobell et al., 1995; Schneider and de Bruijn, 1996; Laguerre et al., 1996 and Vinuesa et al., 1998) have used this highly discriminative and reproducible technique to study the genetic diversity of rhizobial strains.

1.3.2 Characterization of rhizobial isolates using Amplified Ribosomal DNA Restriction Analysis (ARDRA)

The conservative nature of the ribosomal (r)DNA and proteins reflect their essential role in translation processes. The genes encoding the rDNA in the bacteria are frequently organized into an operon (Woose et al. 1990), Figure (1). As shown in this figure, the rDNA operon of most prokaryotes has three main parts. Genes coding for the 16S rDNA (rrs), the 23S rDNA (rrl) and the region between them, named internally transcribed spacer (ITS). The characterization and identification of new isolates as a particular genus or species is based on the variations of chromosomal positions or the structure of rDNA, defined as ribotyping. The techniques of ribotyping has gained popularity because the methods have been proven to be discriminatory and reproducible (Schmidt, 1994).

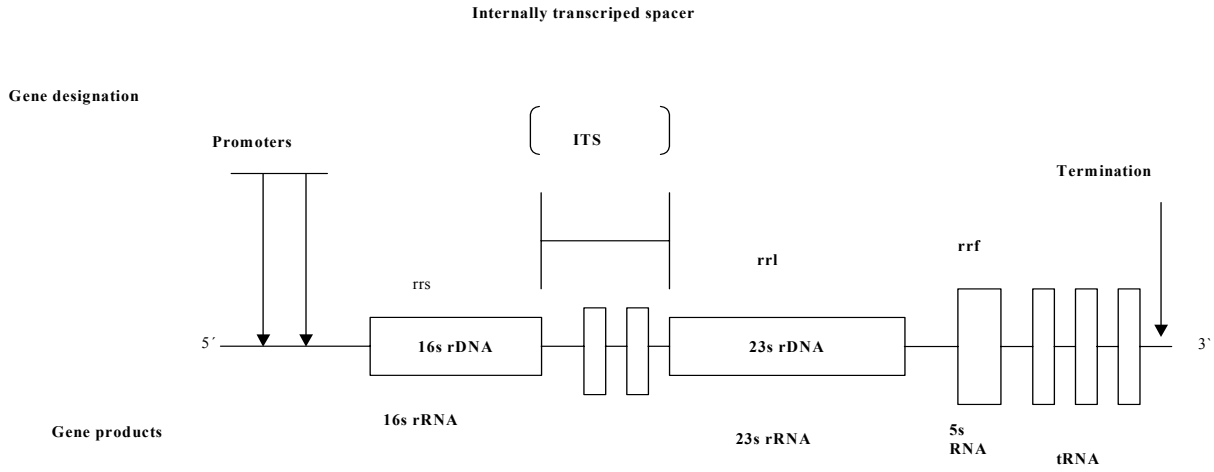


Figure 1. Structure of the ribosomal (rDNA) operon in many prokaryotes.

Three sets of primers are designed to amplify the entire rDNA of prokaryotes, primers fD1 and rD1 (Weisburg et al., 1991) specific for the rrs region, P3 and P4 (Terefework et al., 1998) for genes encoding rrl loci and primers FGPS1490 and FGPL132 for the internal transcribed spacers (Navarro et al., 1992).

RFLP analysis for rDNA genes has been used successfully to identify numerous and phylogenetic diverse bacterial genera (Laguerre et al., 1994; Laguerre et al., 1996; Santamaria et al., 1997;Terefwork et al., 1998; Vinuesa et al., 1998; Rodriguez-Navarro et al., 2000; Lakzian et al., 2002; Odee et al., 2002 and Sliva et al., 2003).

1.3.3 Typing of rhizobial isolates according to their plasmid profiles

Strains of fast growing *Rhizobium* usually contain several large plasmids, some of them exceeding 1mb, are called megaplasmids. These

extrachromosomal elements can make up 50% of the rhizobial genome and can harbor genes essential for survival, competition and symbiotic interaction with host legumes (Garcia de los Santos and Brom, 1996).

As reported by Barbour and Elkan (1989) *R. leguminosarum* bv. *phaseoli* and *R. fredii* contain large plasmids varying in size from 100 to 300 MD that carry genes involved in nodulation (*nod*), symbiotic nitrogen fixation (*fix*) as well as nitrogenase structural genes (*nif*H, D, and K). Plasmids vary in their size according to the species of *Rhizobium*. *Rhizobium* strains may carry from 1 to 10 plasmids (Thurman et al., 1985).

Plasmid profile analysis proved to be a useful tool for differentiating *Rhizobium* strains and studying their genetic diversity (Weaver et al., 1990; Hartman and Amarger, 1991; Zahran, 1992; Brom et al., 1992; Kuykendal and Hashem, 1994; Mhamdi et al., 1999; Brom et al., 2000; Lakzian et al., 2002 and Castro et al., 2003.

1.3.4 Identification of rhizobial isolates using 16S rDNA sequencing and phylogenetic tree analysis

Analysis of 16S rDNA sequences is widely used in modern rhizobial taxonomy, (Young et al., 1991; Laguerre et al., 1993; Segovia et al., 1993; Ludwig et al., 1998 and Sawada et al., 2003). Two types of regions have been distinguished in the 16S rDNA sequence. Type I are the highly conserved motifs which are used to define the relationships among distant taxa, and type Π are the variable sequences which can be used to discriminate between genera and species (Dams et al., 1988). Phylogenetic trees based on rDNA sequence data roughly reflect the evolutionary history of the organisms as shown by comparative sequence analyses of alternative non-rDNA phylogenetic marker molecules (Ludwig and Schleifer, 1994)). Comparative analysis of comprehensive data bases of bacterial 16S rDNA sequences with appropriate software allows rapid identification of unknown rhizobial isolates (Maidak et al., 1994 and Van de Peer et al., 1994). The 16S rDNA sequence analyses based on

phylogenetic trees is a suitable technique to characterize and to study the genetic relationships among *Rhizobium* strains (Sawada et al., 1993, 2003 and Nour et al., 1994). Many rhizobial strains and isolates can be assigned to phylogenetic groups by full or partial 16S rDNA sequence analyses (Dupuy et al., 1994; Van Berkum et al., 1994; Van Rossum et al., 1995 and Amarger et al., 1997).

1.3.5 Hybridization with *nifH* gene

Hybridization with *nifH* gene is an important technique to classify the *Rhizobium* strains within biovars based on the copy numbers of this gene (Segovia et al., 1993 and Amarger et al., 1997).

1.4 Factors that limit the response of rhizobial inoculation in the field:

1.4.1 Biotic factors and competition between indigenous and inoculant rhizobial strains

The majority of soils contain high numbers of indigenous rhizobial strains which are often ineffective in symbiosis but highly competitive due to their adaptation of environmental conditions. The persistence of these indigenous strains in the soil usually limits the response of inoculation with inoculant rhizobial strains. Competition is defined as the ability of a given strain to infect a legume host and form nodules in the presence of other strains. Strains that dominate nodules are considered more competitive than others. The success of inoculation requires that, the inoculum strain must be both highly effective in nitrogen fixation and highly competitive against the native strains (Williams and Phillips, 1983; Singleton and Tavares, 1986 and Segovia et al., 1991). To evaluate competitiveness, it is necessary to identify a specific strain of rhizobia within the nodules. Several different techniques have been used to determine nodule occupancy including fluorescent antibodies (Schmidt et al., 1968), antibiotic resistance (Turco et al., 1986), plasmid profiles (Shishido and Pepper, 1990) and specific gene probes (Judd et al., 1993 and Richardson et al., 1995). DNA markers as the *Gus* gene are especially useful techniques to mark rhizobial

strain for studying the competitiveness (Wilson et al., 1991). The *Gus* operon codes for glucuronidase and it is used as a DNA marker for detecting of bacteria in the rhizosphere or in association with plants (Wilson et al., 1991).

Bean nodules infected by rhizobial strains carrying the *Gus* gene are staining blue when incubated with x-gluc buffer (Wilson et al., 1991). Many investigators (Streit et al., 1992; Wilson, 1995; Wilson et al., 1995; Streit et al., 1995; Sessitsch et al., 1997; Sessitsch et al., 1998; Anyango et al., 1998; Diouf et al., 2000 and Denton et al., 2003) have used this technique to identify rhizobial strains inside nodules after inoculation with competing strains.

1.4.2 A biotic factors:

1- Salinity of soil

Nearly 40% of the world land surface can be categorized as having potential salinity problems affecting soil fertility (Cordovilla et al., 1994). Most of these areas are confined to the tropics and the Mediterranean regions. The ability of legume crops to grow under stress of salt varies greatly and depends on such factors as climate conditions, soil properties and crop cultivars (Cordovilla et al., 1995). Variability in salt tolerance among legume crops has been reported (Zahran, 1991 and 1999). Legumes such as *Phaseolus vulgaris*, *Vicia faba* and *Glycin max* are more salt tolerant than *Pisum sativum* (Cordovilla et al., 1995). The legume plants are more sensitive to salt or osmotic stress than the rhizobia (Elshinnawi et al., 1989; Velagaleti et al., 1990 and Zahran and Sprent, 1986). Although the root-colonizing bacteria of the genera *Rhizobium* and *Bradyrhizobium* are more salt tolerant than their legume hosts, they also show marked variation in salt tolerance. Growth of rhizobia was inhibited by 100 mM NaCl (Yelton et al., 1983). Fast growing rhizobia are more salt tolerant than slow growing species (Elsheikh and Wood, 1995). *Rhizobium* nodulating *Phaseolus vulgaris* and isolated from Moracco were able to resist a sodium chloride concentration up to 4% NaCl (680 mM NaCl) in liquid culture (Priefer

et al., 2001). The effects of salt stress on nodulation and nitrogen fixation of legumes have been examined in several studies (Delgado et al., 1994; Moawad and Beck 1991; Elshinnawi et al., 1989; Igual et al., 1997; Nair et al., 1993 and Subba Rao et al., 1990). Salt stress inhibits the initial steps of *Rhizobium*-legume symbiosis. Soybean root hairs did show little curling or deformation when inoculated with *Bradyrhizobium japonicum* in the presence of 170 mM NaCl and nodulation was completely suppressed at 210 mM NaCl (Tu, 1981). The reduction of N_2 fixing activity by salt stress is usually attributed to a reduction in respiration of nodules and reduction in cytosolic protein production specially leghemoglobin by nodules (Delgado et al., 1994). High salt concentration resulted in decreasing nodulation and amounts of N_2 fixed (Bekki et al., 1987). The successful of *Rhizobium*-legume symbiosis under salt stress requires a good selection for both salt tolerant rhizobial strains (Zahran, 1991) and host plants (Saadallah et al., 2001).

Two different strategies exist within microorganisms to survive under high salt concentrations. First, cells may maintain high intracellular salt concentration that should be equivalent to the external concentration (salt-in strategy). With this strategy all the intracellular systems should be adapted to the presence of high salt concentration. Second, cells may keep a low salt concentration within their cytoplasm (compatible solutes strategy). The osmotic pressure of the medium is balanced by the production of compatible solutes (Oren, 1999).

2- Soil pH

Soil acidity adversely affects survival, growth and the capacity of nitrogen fixation of rhizobial strains (Lie, 1981). The low pH of soil affects all stages of the legume-*Rhizobium* symbiosis, including strain survival in the soil, root hair infection, nodule initiation and nitrogen fixation (Graham et al., 1982; Wood et al., 1984). Soil acidity is a significant problem for agricultural production in many areas of the world and limits legume productivity (Bordeleau and Prevost,

1994; Graham, 1992). The failure of nodulation was reported in legumes especially in the acid soil below pH 5 due to the inability of *Rhizobium* strain to survive under these acid conditions (Graham et al., 1982; Carter et al., 1994). There are also many reports about nodulation and nitrogen fixation in acid soils (Lie, 1981; Graham et al., 1982; Wood et al., 1984; Graham, 1992; Bordeleau and Prevost, 1994 and Carter et al., 1994) but only a few studies on the effects of alkaline soils (Surange et al., 1997 and Kulkarni et al., 2000). Highly alkaline soils of pH above 8 tend to be high in contents of sodium chloride, bicarbonate and borate which reduce nitrogen fixation (Bordeleau and Prevost, 1994). High pH completely inhibited nodulation of some lupines (Tang and Robson, 1993).

3- Soil temperature

Elevated temperatures in tropical soils are a major problems restricting the response of inoculation with introduced or indigenous rhizobial strains especially in the summer season (Michiels et al., 1994). High soil temperature is associated with delaying or restricting nodulation in the subsurface region (Graham, 1992). Temperature affects several stages of symbiosis such as root hair infection, bacteroid differentiation, nodule structure and functioning (Roughley and Dart, 1970). Nodule functioning in common beans (*Phaseolus Spp*.) is optimal between 25 and 30 $^{\circ}$C, but is hampered by root temperatures between 30 and 33 $^{\circ}$C (Piha and Munns, 1987). Bean nodules formed at 35 $^{\circ}$C are very small and have low specific nitrogenase activity. Michiels et al. (1994) found that the acetylene reduction activity of common bean plants was strongly diminished at 35 $^{\circ}$C when plants were inoculated by heat-sensitive or heat-tolerant strains. Surange et al. (1997) and Kulkarni et al. (2000) isolated highly temperature (50 $^{\circ}$C) tolerant strains of *Rhizobium* that nodulating legume trees from tropical soils.

4- Availability of nitrogen in the soil

It is widely accepted that the capacity for nitrogen fixation by nodulated legumes is influenced, by mineral nitrogen in the soil at least in two ways. First, the process of nodulation may be promoted by relatively low levels of available nitrate or ammonia, second, higher concentrations of nitrogen always depress nodulation (Eaglesham, 1989). Danso et al. (1990) found that the inhibition of soybean N_2 fixation at higher N levels (83 mg of N kg of $soil^{-1}$) was significantly reduced. In *Rhizobium*-legume symbiosis, the formation of nodules is the result of a complex multi steps process. Combined N is a major environmental factor limiting the development and success of the *Rhizobium*-legume symbiosis in the field (Danso et al. 1990 and Munns, 1968). The inhibitory effect of nitrate on N_2 fixation has been attributed to a direct competition between nitrate reductase and nitrogenase for reducing power (Straub et al., 1997) or to the hypothesis that nitrite as intermediate of nitrate reductase inhibits the function of nitrogenase or leghemoglobin (Becana and Sprent, 1987).

1.5 Genes involved in salt tolerance

The response and adaptation of rhizobial strains to environmental stresses involves many physiological and biochemical processes linked to differential gene expression. Botsford (1990) reported that the production of 41 proteins was increased at least 10-fold in salt-stressed cells of *Escherichia coli*. *R. tropici* CIAT899 has been shown to tolerate several environmental stresses, including salinity, low pH and high temperature (Graham et al., 1994). Eight different genes loci in a salt tolerant strain of *R. tropici* CIAT 899 were identified (Nogales et al. 2002) when grown under stress of salinity. These genes could be classified into three groups: the first group are two genes involved in the regulation of gene expression, such as ntrY and ntrX, two components regulatory system involved in regulation of nitrogen metabolism. The second group are genes related to synthesis, assembly or maturation of proteins, as alaS

coding for alanine-tRNA synthetase, dnaJ encoding a molecular chaperone and a nifS homolog, probably encoding a cysteine desulfurase involved in the maturation of Fe-S proteins. The third group are genes involved in cellular build up such as noeJ responsible for producing mannose-1-phosphate guanylytransferase which plays an important role in lipopolysaccharide biosynthesis and kup genes for an inner-membrane protein involved in potassium uptake. Survival and growth in saline environments are the result of adaptive process, such as ion transport and compartmentation, osmotic solute synthesis and accumulation, which lead to osmotic adjustment and protein turnover for cellular repair (Rai et al., 1985). Several chemical substances from micro-organisms and plants were reported to be accumulated under salt stress to protect themselves against this kind of stress such as, polyamines (Aziz et al., 1999; Kasinathan and Wingler, 2004), polysaccharides (Rüberg et al., 2003), amino acids (TeChien et al., 1992), organic solutes (Gilbert et al., 1998; Soussi et al., 1998; Sairam and Tyagi, 2004), soluble sugars (Hu and Schmidhalter, 1998; Sairam and Tyagi, 2004) and inorganic cations as K^+ (Asch et al., 1999; Smith and Smith, 1989). High production rates of cytoplasmic K+, organic solutes and glycine betaine could be correlated with the ability of rhizobial strains to resist salt stress (Galinski, 1995) as well as dipeptides (N-acetyl-glutaminylglutamine amide) as reported by Smith et al. (1997). The reduction in amino acids biosynthesis was also reported by TeChien et al. (1992) and Rüberg et al. (2003). Cavalcanti et al. (2004) and Tejera et al. (2004) reported the reduction of catalase under salt stress in cowpea leaves and in nodules formed by *R. tropici* on the roots of *Phaseolus vulgaris*. Putative oxidoreductase proteins were detected only in nodule bacteroid phase of *Sinorhizobium meliloti* but were not detected in free living cells (Djordievic, 2004). Bolaños et al. (2003) found that, salt stress was contributed to damage or alter the nodule cell walls of *Pisum sativum*.

2 Materials and Methods

2.1 Isolation of rhizobia from bean nodules, and standard strains

New strains were isolated from nodules after surface sterilization. Nodules collected from the field, were first washed in tap water several times and vortexed in 0.05% (v/v) Tween 20 to remove adhering particles of soil. After that nodules were surface sterilized according to the procedure described below.

1- Rinse the nodules in 70% (v/v) ethanol for 30 sec, followed by a 3% (v/v) NaOCl.
2- Wash the nodules thoroughly in sterile tap water at least five times.
3- Crash nodules in 50 µl YM broth or 20E medium.
4- Streak a loop full of the resulting suspension on to YM or 20E plates and incubate them for 2-3 days.

Nodules collected from plants grown in the growth pouches or in leonard jars are surface sterilized by dipping into 95% ethanol for five sec and briefly flaming them. Isolates and their location are listed in Table 1, standard strains and plasmid are listed in Table 2.

Table 1. Origin of isolates in Egypt, soil characteristics and strain designation.

Locations	**Total number of isolates**	**Soil type**	**pH**	**Electric conductivity EC (m sm^{-1})**	**designation**
Isma'ilya	5	Sandy loam	8.0	150	EBRI 2, 3, 6, 25 and 29
Ashmun	7	Clay	7.2	130	EBRI 20, 21, 23, 24, 26, 27 and 32

EBRI: Egyptian bean rhizobial isolates.

Table 2. Standard strains and source of plasmids.

Strain	**Source**
Rhizobium tropici CIAT 899	Martinez-Romero et al., 1991
Rhizobium etli CFN42	Segovia et al., 1993
Agrobacterium tumefaciens 30150	DSM
E. coli S17-1-λ-pir (pCAM130)	De Lorenzo et al., 1990
Rhizobium gallicum R602sp	Amarger et al., 1997
Rhizobium giardinii H152	Amarger et al., 1997
Sinorhizobium meliloti 2011	Dr: Bohlool

YM medium (Somasegaran and Hoben, 1985).

K_2HPO_4	0.5g
$MgSO_4x7H_2O$	0.3g
NaCl	0.1g
Yeast extract	0.5g
Mannitol	10g
H_2O	up to 1000 ml
pH	6.8-7

20E medium (Werner et al., 1975).

KH_2PO_4	86mg
K_2HPO_4	87mg
$MgSO_4x7H_2O$	370mg
$CaCl_2x2H_2O$	73.5mg
KNO_3	506mg
$FeSO_4xH_2O$	6.95mg
EDTA	9.3mg
$Na_2MoO_4x2H_2O$	4.84mg
Glycerol	4.6ml
Mannitol	1.82mg
Yeast extract	2g
H_2O	up to 1000 ml
pH	6.8-7

2.2 Isolation of genomic DNA from rhizobial strains

Total or genomic DNA from rhizobia and other gram negative bacteria obtained from 1.5 or 2 ml of liquid cultures grown to the middle of exponential phase was isolated by the standard method of cetyltrimethyl-ammonium bromide (CTAB) protocol, as described by Ausubel et al. (1994). This protocol is particularly suited for strains that produce large amount of exopolysaccharides into the growth medium as rhizobial strains do. An outline of the method is given below:

1- Harvest 2 ml of bacterial culture at the middle of exponential phase by centrifugation at 14000 rpm, and remove the supernatant as completely as possible.
2- Resuspend the cell pellet to homogeneity in 756 µl TE buffer.
3- Add 40 µl of 10% SDS and 4 µl of stock solution of (20 mg ml^{-1} proteinase K) and incubate for at least 1 h at 37 ^{0}C for cell lysis.
4- Add 133.3 µl of 5 M NaCl, and mix well.

5- Add 107µl of CTAB-NaCl solution, mix thoroughly and incubate 10 min at 65 °C.
6- Extract the complex of CTAB-protein-polysaccharides by shaking with one volume of (900-1000 µl) Chloroform/Isoamyl alcohol (24:1; v/v) and 4-5 min spinning in the centrifuge.
7- Remove the aqueous, viscous phase to a new microcentrifuge tube, leaving the interface behind, and extract once with one volume of Phenol: Chloroform: Isoamyl alcohol (25:24:1; v/v/v).
8- Centrifuge at maximum speed for 5 min, remove the aqueous phase to a new tube and precipitate the pellet of DNA by adding 0.6-0.7 volume isopropanol and rapid flicking of the tubes. The DNA is usually seen as white threads, which are sediment by a short centrifugation.
9- The DNA pellet should washed two times with 70% Et, dried in air under a Laminar flow and dissolved in 50 µl of T.E. and stored at 4 °C. To check the purity of DNA, 6 µl of DNA loaded on 0.8% mini- agarose gel for 20 min and the gel soaked for 30 min in EtBr, and detected under UV light.

Buffers
TE buffer: Tris-HCl 10 mM, EDTA 1 mM, pH 8.
NaCl 5M: 292.5 g NaCl l^{-1}**; SDS** 10% (w/v); **Proteinase K** (20 mg ml^{-1} stocks at –20 °C).
NaCl-CTAB solution: dissolve 4.1 g NaCl in 80 ml H_2O, slowly add 10 g CTAB while stirring, warm them up to 65 °C and complete to 100 ml with distilled water.
Chloroform:isoamyl (24:1 v/v)
Phenol: chloroform:isoamyl (25:24:1 v/v/v).

2.3 Estimation of DNA concentration

For the DNA concentration and quality, the absorption spectra between 220 and 300 nm was recorded. A small amount of DNA was used to measure the absorption value at 260 nm and 280 nm using GeneQuant Π photometer. The absorption ratio at the two wave lengths should be between 1.8 to 2 in order to have a good quality of DNA (Sambrook et al., 1989).

2.4 Methods of Molecular characterization

2.4.1 REP-PCR genomic finger printing

Repetitive sequence-based genomic fingerprints were generated using primers REPIR and REP21 (Table 3) in 25 µl PCR mixture as shown in Table A1 and cycling parameters as described in Table 4. Six µl of the products were loaded onto 18 cm long 1.5% agarose gels and run in TAE buffer pH8 for 4.2 h at 4 v/cm. A 1Kb ladder (GIBCO-BRL) was used for the normalization of the pattern.

TAE buffer: 2M Tris-HCl (pH 8), 1M acetic acid, 50 mM EDTA.

2.4.2 Amplified Ribosomal DNA Restriction Analysis (ARDRA)

Primers fD1 and rD1 (Table 3) were used to amplify nearly full-length 16S rDNA genes as explained by Weisburg et al. (1991), while primers P3 and P4 were used to amplify the region of 23S rDNA as described by Terefework et al. (1998). The PCR reaction was performed using the standard reaction mixture (50 µl in Table A2) and cycling parameters as reported in detail in Table 4. The amplified products of 16S rDNA were digested with four restriction analysis enzymes, *HhaI*, *DdeI*, *MspI* and *Sau3aI*, while the 23S rDNA amplicons were restricted with enzymes *HinfI*, *Sau3aI* and *MspI*, (USB Amersham International), as recommended by the manufacturer.

The 16S-23S rDNA Internal Transcribed Spacer region was amplified with primers FGPS1490 and FGPL132 (Navarro et al., 1992) using the standard PCR mixture (see Table A2) and cycling conditions described in Table 4.

Table 3. List of primers used to amplify REP, entire rDNA, 16S rDNA sequencing and *nodC* genes.

Name of primers	**Used to amplify**	**Sequence**	**Reference**
REPIR	REP	5'-ΠΠCGI CGI CAT CIG GC-3'	Versalovic et al., 1994
REP21	REP	5'- ICG ICT TAT CIG GCC TAC-3'	Versalovic et al., 1994
rD1	16S rDNA	5'-AAG GAG ATC CAG CC-3'	Weisburg et al., 1991
fD1	16S rDNA	5'-AGA GTT TGA TCC TGG CTC AG-3'	Weisburg et al., 1991
P3	23S rDNA	5'- CCG TGC GGG AAA GGT CAA AAG TAC C-3'	Terefework et al., 1998
P4	23S rDNA	5'- CCC GCT TAG ATG CTT TCA GC-3'	Terefework et al., 1998
FGPS1490	ITS	5'-TGC GGC TGG ATC ACC TCC TT-3'	Navarro et al., 1992
FGPL132	ITS	5'- CCG GGT TTC CCC CAT TCG G-3'	Navarro et al., 1992
nodcF2	*nodC*	5'-AYG THG TYG AYG ACG GCT – C'	Laguerre et al., 2001
nodcI	*nodC*	5'-CGY GAC AGC CAN TCK CTA TTG-3'	Laguerre et al., 2001
Internal primers	16S rDNA sequencing	5'- CCG GGC GGT GTG TAC AGG G-3' 5'- TAC GGG AGG CAG CA G-3' 5'-GTC AAT TCC TTT GAG-3' 5'-CCG GGC GGT GTG TAC AAG G-3'	Amarger et al., 1997

Table 4. Cycling parameters for REP, 16S rDNA, 23S rDNA, ITS and *nodC* .

REP	**16S rDNA and 23S rDNA**
95°C 2min 93°C 45sec ← X30 40°C 1min 65°C 8min 4°C	95°C 2min 94°C 15sec ← X10 93°C 45sec 55°C 45sec 72°C 2min 94°C 15sec ← X20 93°C 45sec 55°C 45sec 72°C 2min+1sec/cycle 72°C 5min 4°C
ITS rDNA	***nodC***
95°C 3min 94°C 15sec ← X30 93°C 45sec 55°C 40sec 72°C 1min 72°C 5min 4°C	37°C 30sec ← X5 60°C 2min 94°C 30sec 50°C 3osec 72°C 30sec ← X27 94°C 30see 4°C

All restriction patterns were resolved on a 2% agarose gel electrophoresis system in TBE buffer at 55v for 3h. The RFLP patterns were normalized using a 100 bp ladder from GIBCO BRL (Eggenstein, Germany). The gels were stained post-electrophoresis with EtBr. ITS products were not digested with restriction enzymes because some of the examined strains gave two amplified fragments.

2.4.3 Analysis of REP-PCR fingerprints and rDNA restriction patterns

Gel images were digitised using a charge coupled device video Camera (INTAS, Göttingen, Germany) and stored as TIEF files. Data were analysed using the Gel Compare Π software package (version 2.0; Applied Maths, Kortrijk, Belgium) as described in detail by Viunesa et al., 1998).

2.4.4 Amplification of *nodC* genes

Primers nodCF2 and nodCI (Table 3) were used to synthesize the *nodC* fragment from rhizobial isolates and standard strains as used by Laguerre et al., (2001). The PCR reaction was performed using the standard reaction mixture (50µl in Table A2) and cycling parameters as mentioned in Table 4.

2.4.5 Plasmid DNA content

Plasmid profile analysis for twelve rhizobial isolates EBRI 2, 3, 6, 20, 21, 23, 24, 25, 26, 27, 29, and 32 in addition to *R. tropici* CIAT 899, *R. gallicum* R602sp and *R. giardinii* H152 as standard strains and *R. etli* CFN 42 as reference strain in order to estimate the size of generated plasmids, was done according to the method described by Kuykendall and Hashem (1994):

1- *Rhizobium* strains were grown for 18-24 h at 30 °C in an incubator shaker at 100 rpm.
2- Portions of 0.2-0.3 ml culture were put into a sterile eppendorf tube on ice.
3- 0.3 % sarkosyl (N-lauroyl sarcosine) in 1x TBE buffer was added, and mixed gently by tapping, and pelleting the cells by centrifugation at 17,000 rpm at 4 °C for 5 min.
4- After keeping them on ice for 10 min, remove the supernatant completely, without disturbing the pellet and resuspend the pellets with 25 µl lysis buffer by tapping, then immediately the mixture should be loaded on 0.75% agarose gel containing 1% SDS.
5- Run the samples at 5 v for 30-45 min until the turbidity disappeared from the wells, then at 85 v for 8 h.
6- The gels were stained with EtBr at room temperature for 30 min.

Buffers

TBE: Tris 218 g, EDTA 18 g, H_3BO_3 111.3 g and distilled water 2 litter, pH 8.3.
0.3% N-lauroyl sarcosine: 0.3 g Sarcosine in 100 ml TBE buffer.
Lysis buffer: 0.2 mg ml $^{-1}$lysozyme in 10% sucrose, 10 μg ml^{-1} RNase in 1x TBE.

2.4.6 Direct sequencing of 16S rDNA

The amplified fragment of 16S rDNA was purified using QIAGEN kit according to the instructions of the manufacturer. To read the whole sequence of the fragment, primers rD1 and fD1 in addition to four internal primers (Table 3) were used. A DNA sequencer machine 373A and the dye terminator cycle sequencing kits with AmpliTaq-DNA-polymerase from Applied Biosystems were used, based on the enzymatic chain terminator technique developed by Sanger et al. (1977). The cycling program was:

Steps	Denaturation	Annealing	Extension	Cycles number
1	96°C 1min	-	-	1
2	95°C 40sec	55°C 40sec	72°C 4min	25
3	-	-	72°C 4min	1

a- Preparation of sequencing gels

Six percent polyacrylamide gel were prepared by mixing 24 ml of Sequagel™ XR and 6 ml Sequagel™ with 280 μl of 10% (w/v) APS. This solution was poured into the previously assembled 25x40 cm long gel casting chamber with 0.25 mm thick spacers. After polymerisation, and before loading the samples, a pre-run of about 20 min was made at 1500V, 25 mA and 30 W, until the gel reached 50 °C. Signal auto gain and scanner focusing were performed as recommended by the manufacturer.

b- Comparing the sequence analysis based on computer programs

DNA-STAR software was used for sequence editing. For searching to find the homologies of sequences from Databank the BLASTN program was used. The 16S rDNA sequence for EBRI isolates were deposited in the Gene Bank under accession numbers AY 221174 to AY 221181.

c- 16S rDNA sequence alignment and phylogeny estimation

Sequence similarity searches were performed at the NCBI server using BLASTN (http://www.ncbi.nlm.nih.gov/blast). Multiple nucleotide sequence alignments were generated and edited using ClustalW, as implemented in Bio Edit (Hall, 1999). The rrs multiple sequence alignment was manually adjusted to fit that produced by the Ribosomal Database Project-Π (Maidak et al., 2001). Model fitting was performed by likelihood ratio tests (LRTs) as implemented in DAMBE (Xia and Xie, 2001). A neighbour joining (NJ) phylogeny was inferred with the model selected by LRTs using MEGA2.1 (Kumar et al., 2001) and the complete gap deletion option. The robustness of the phylogeny was assessed by non-parametric bootstrapping with 1000 pseudoreplicates.

2.4.7 Transfer of DNA from agarose gels to nylon membranes (Southern-blot)

DNA can be easily transferred by different methods from agarose gel to the nylon membrane. One of these methods is the vacuum blotting systems (Southern, 1975). Genomic DNA digested with restriction enzymes and fractioned on 0.8% (w/v) agarose-TAE gel were moved to the nylon membranes (Boehringer-Mannheim, Germany) using the vacuum system (Vacu Gene XL:Pharmacia, Germany). The gel is placed on top of the filter, which was previously placed on the blotter and surrounded by an adequate plastic foil to ensure, that the vacuum can be generated with an appropriate pump. The DNA

is first denatured in the gel by covering it with denaturation solution (1.5M NaCl, 0.5M NaOH) and application of 35 mbar vacuum for 20 min, followed by neutralization with 1 M ammonium acetate at the same pressure and incubation time. To move the DNA to the nylon membrane, it is essential to increase the vacuum to 45 mbar for 30 min, with addition of fixation solution (1M ammonium acetate). After transferring the DNA, it needed to be fixed by exposing the nylon membrane to the UV cross linker using a UV-cross linker (Stratagene). The filter should kept at –20 °C until further hybridization.

2.4.8 Generation of digoxigenin-labelled *NifH*-DNA probe

Southern blot hybridization experiment was done using digoxigenin (DIG) labelled DNA probe. This labelling system is based on the insertion of alkali labile DIG-dUTP into the novel synthesized DNA strands. The labelling reaction was usually performed in 50 µl reaction volumes (Table A3) and 3-5 µg of template DNA. To obtain the probe of *nifH* gene, primers nifHF (5'-TAC GGN AAR GGS GGN ATC GGC AA-3'), nifHI [5'-AGC ATG TCY TCS AGY TCN TCC A-3' (Laguerre et al., 2001)] and DIG-labelling kit from (Boehringer-Mannheim, Germany) were used. The cycling program was used as for the standard PCR amplification of the target fragment. *NifH*-DIG labelled probe can be easily recognized from the gel by running the PCR product on 0.8% agarose in TAE buffer. DIG-labelled PCR-fragment should have lower relative mobility on the gel due to the insertion of digoxigenin molecule compared with the unlabelled fragment faster in the mobility (Figure 2).

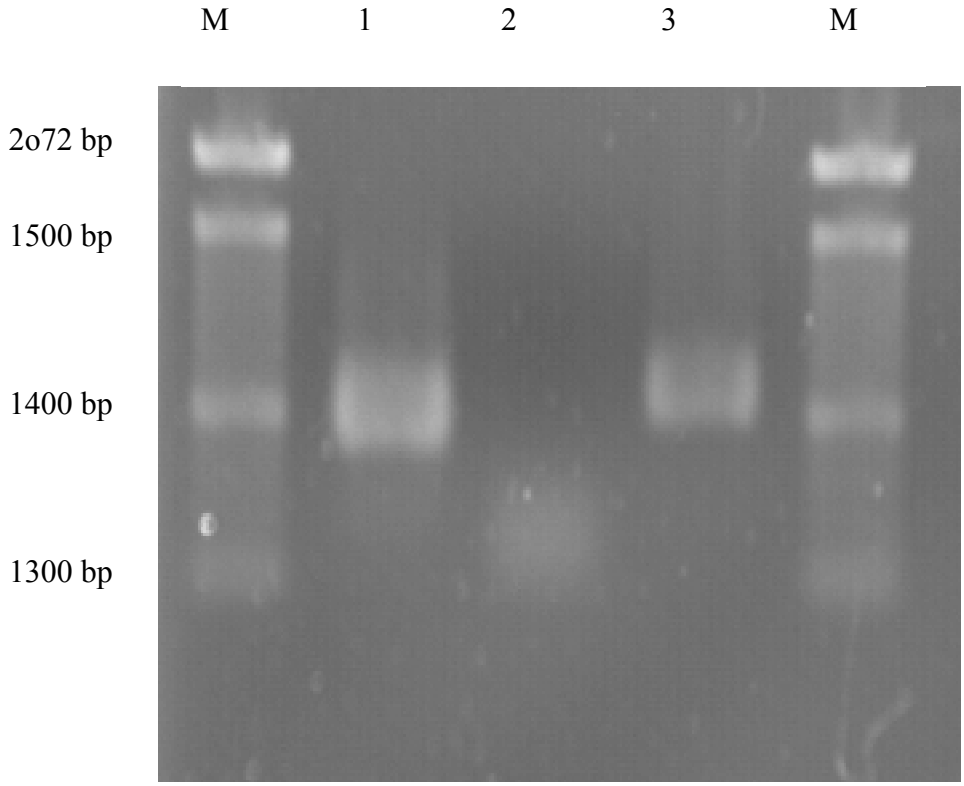


Figure 2. nifH-DIG labelling probe obtained from the PCR reaction, Lanes 1 and 3 show the DIG-labelled fragments of nifH gene, lane 2 show the faster migration fragment (unlabelled) and M is the 100 bp ladder.

2.4.9 Southern hybridization with DIG-labelled DNA probe and signal detection

Prehybridization, hybridization, stringency washes, incubation of probed filter with antidigoxigenin Fab fragments coupled with alkaline phosphatase and detection of alkaline phosphatase activity of the filters were done according to the protocols in the DIG System (User's Guide for Filter Hybridization) from Boehringer-Mannheim, Germany.

The protocols are as follows:

Prehybridization and hybridization

1- Prehybridize membranes with fixed DNA for 1h in the hybridization solution, in the hybridization oven.
2- Remove the hybridization solution and add the heat-denatured probe dissolved in the hybridization solution, (generally a PCR labelled probe specific for the fragment to be detected and randomly labelled marker

DNA) incubate overnight in the hybridization oven (the probe can be used three times when removed and kept at –20 °C).

Post hybridization washes and membrane blocking

3- Remove the excess of the probe by washing twice at room temperature (rt) with 2x SSC for 5 min, followed by washing twice for 15 min with 0.5x or 0.1x SSC at 65 °C in the hybridization oven.
4- Equilibrate the membranes in washing buffer for 1 min.
5- Incubate the filter in 1x blocking solution at rt with gentle agitation for 30 min.

Incubation of membranes with anti-DIG-AP Fab and colorimetric detection signals with NBT and x-phosphate

1- Pour off the blocking solution and incubate the membrane with anti digoxigenin (Fab) fragments conjugated with alkaline phosphatase [1: 10000 (75mU ml^{-1}) in 1x blocking solution], for 30 min at rt.
2- Eliminate the excess of antibody solution and wash gently twice with washing buffer for 15 min at rt.
3- Discard the washing buffer and equilibrate the filter in detection buffer for 2 min at rt.
4- Prepare solution mix of 45 µl NBT and 35 µl of X-phosphate in 10 ml of detection buffer. Added the freshly prepared mixed solution to the membrane and seal the hole system in a plastic bag and incubate it in the dark without shaking.
5- Stop the colour reaction by removing the solution mix and washing the membrane with TE buffer. Note, that the filter can be kept with TE buffer at 4 °C.

Solutions:

Standard prehybridization solution: 5x SSC, 1% (w/v) blocking reagent (Boehringer-Mannheim), 0.1 % N-lauroyl Sarcosine, 0.02% SDS.
20x SSC: 3 M NaCl, 300 mM Sodium citrate, pH 7.
Malic acid buffer: 150 mM NaCl, 100 mM malic acid, pH 7.5.
Washing buffer: 0.3% Tween 20 in malic acid buffer.
10x Blocking solution: 10% (w/v) blocking reagent in malic acid buffer, dissolve by stirring on a heat plate without boiling.
NBT and x-phosphate solution: Dissolve 75 mg ml^{-1} nitroblue tetrazolium salt (NBT) in 70% (v/v) DMF and 50 mg ml^{-1} 5-bromo-4-chloro-3-indolyl phosphate salt (BCIP or X-phosphate) in 100% DMF.
Detection buffer: 0.1 M tris-HCl, pH 9.5; 0.1 M NaCl, 0.05 M $Mg\ Cl_2$
(Note: prepare the 0.1 M Tris-HCl pH 9.5 solution from a 1 M stock solution to avoid precipitation of $MgCl_2$).

2.5 Nodulation experiments

2.5.1 Cultivars of common bean (*Phaseolus vulgaris*)

Three bean cultivars were used in this study. Cultivars Saxa and Canoca from Germany and Colombia were obtained from the collection of germplasm in the Department of Biology, Philipps-University Marburg, the cultivar Giza 6 was obtained from the Institute of Vegetables Crops Research, Ministry of Agriculture, Egypt.

2.5.2 Seeds germination and sterilization

Seeds of *Phaseolus vulgaris* and *Leucaena leucocephala* were surface sterilized based on the method described by (Vinuesa et al., 1998) as explained below:

1- Wash the seeds with tap water and a few drops of Tween 20.
2- Rinse the seeds for 30 sec in 70% et.
3- Soak the seeds for 3 min in NaOCl.
4- Wash the seeds several times with sterile water.
5- Leave the seeds in sterile water for 1 h after removing the excess of sodium hypo chlorite on a shaker to imbibe.
6- Wash the seeds again for two times with sterile water and distribute them on 1% agar plates and incubate them for 2-4 days.

2.5.3 Plant nodulation assays

The nodulation assays were performed in sterilized growth pouches or in Leonard jars with vermiculite: perlite (1:1v/v) as a medium for cultivation and N-free nutrient solution (Table 5). Seeds of common bean were surface sterilized and distributed on the surface of 1% agar plates, then incubated for 3-4 days at 28 $^{\circ}$C for germination. After germination, seedlings were transferred to growth pouches or Leonard jars under sterile conditions. Seeds of *Leucaena leucocephala* cv. Cunningham were scarified for 20 min in concentrated sulphuric acid, then surface sterilized and cultivated as previously described. Seeds were inoculated by adding 1 ml of rhizobial cultures at the middle of the

exponential growth phase with about 10^8 cells ml^{-1} for each Leonard jars. For inoculating the seeds in growth pouches, seeds were inoculated by soaking in one ml of the diluted culture 1:20 with sterile nutrient solution for three minutes. Plants were cultivated in controlled growth chambers with 15h in the light at 25 °C and 9 h in the dark at 18 °C and relative humidity of 75%. Light intensity was 14 k Lux. Plants were harvested 21, 25 or 30 days after inoculation.

Table 5. N free nutrient solution (Werner et al., 1975).

Substance	**Amount**	**Substance**	**Amount**
K_2So_4	279mg	$MnSO_4.4H_2O$	1.02mg
$MgSO_4.7H_2O$	493mg	$ZnSO_4.7H_2O$	0.22mg
KH_2PO_4	23mg	$CuSO_4.5H_2O$	0.08mg
K_2HpO_4	145mg	$Na_2MoO_4.4H_2O$	0.05mg
$CaCl_2.2H_2O$	371mg	$CoCl_2.4H_2O$	0.1mg
H_3BO_3	1.43mg	Fe-EDTA	16.7mg
Distilled water to 1000ml			

2.5.4 Estimation of the acetylene reduction assays (ARA) by Bender and Rolfe (1985)

To measure the activity of acetylene reduction, root systems with nodules were incubated for 1h with 10% acetylene gas in air. After incubation, 0.5 ml of the gas mixture was fractionated by injection into a gas chromatograph with a flame ionization detector. The amount of nitrogen fixed from atmosphere was estimated based on the peak area of ethylene generated as a result of reduction for the acetylene gas by nitrogenase, using the following equation:

nM C_2H_4 $h^{-1}mg^{-1}$ nodules= concentration of ethylene x area of ethylene x volume x 2 ÷ concentration of standard x incubation time x mg nodules, and using a ratio of 4 n mol C_2H_4 produced for 1 nmol N_2 fixed.

2.5.5 Physiological trials to select the strains adapted to environmental conditions

Growth of bean rhizobial strains from Egypt and some of the standard strains was examined in 20E broth culture amended with different NaCl concentration in order to select the strains tolerated to saline soils (Moawad and Beck, 1991). To select the strains resistant to the alkaline soils (pH > 7 to 9), the same medium was buffered with AMPD buffer while the medium buffered with 25 mM HOMOPIPES for pH ranging between 4 and 5. To adjust the medium pH from 5.5 to 7, 20 mM MES was added as described by Priefer et al. (2001). To choose the strains adapted to high temperature, cultures were incubated at 37^0C and 42 °C compared to 30 °C. The growth was recorded as increase in turbidity (OD) compared with the growth of strains under standard conditions.

2.5.5 Plant nodulation assays and symbiotic performance under stress of salinity or alkalinity

Seed sterilization, plant germination and cultivation were performed as done previously. To examine the symbiotic performance of some selected strains under salt stress, sodium chloride was added to the nutrient solution to a final concentration of 0.2% NaCl (34.2 mM) or 0.4% NaCl (64.4 mM). For testing the alkaline stress, the nutrient solution was buffered with AMPD to adjust the medium pH to 8 or 8.5 (Priefer et al., 2001). Plants were cultivated in a controlled growth chamber under the same conditions described before and harvested 21 days after planting and the nodulation parameters were estimated.

2.6 Competition experiments

2.6.1 Construction of a *R. tropici* CIAT 899 *Gus*$^+$ strain

R. tropici CIAT 899 integrated the *Gus* gene from the mobilizable plasmid PCAM130, from the donor strain *E. coli* S17-λ-pir (de Lorenzo et al., 1990) by conjugation (Simon et al., 1983). Three loops of *E. coli* strain S17-λ-pir grown on LB medium (Sambrook et al., 1989) and three loops of *R. tropici* CIAT 899 were mixed and left for patch mating on PY medium plates (Noel et al., 1984), by incubating at 28 °C overnight. Bacteria were transferred into 5 ml of 0.85% NaCl plus 0.05% Tween 20 to remove the excess of exopolysaccharides, and diluted to 10^{-4}. 100 µl of this dilution were plated on MM medium (Kingsly and Bohlool, 1992) amended with 50 µg ml^{-1} sp and str to select the recipient strain of *R. tropici* CIAT 899 with Tn5ssgusA30. Colonies were re-streaked on the MM plus 50 µg ml^{-1} sp and x-gluc substrate to screen for spectinomycin resistant colonies marked with the *Gus* gene. Blue colonies were recognized, picked up and sub-cultured on the same medium to check the purity and the *Gus* stability of the colonies. Colonies marked with the *Gus* gene were kept with 50% glycerol at –70 °C for competition studies.

LB medium (Sambrook et al., 1989)

Tryptone	10g
Yeast extract	5g
NaCl	10g
H_2O	to 1000 ml
pH	7.5

PY (Noel et al., 1984)

Tryptone	5g
Yeast extract	3g
$CaCl_2$	1g
H_2O	to 1000ml
pH	6.8

Minimal medium (Kingsly and Bohlool, 1992)

Glycerol	5.17ml
Na-glutamate	2.09g
$MgSO_4$ x7H_2O	246mg
$CaCl_2$x2H_2O	68mg
KCl	112mg
Fe-EDTA	11mg
KH_2PO_4	685µg
$MnSO_4$ xH_2O	152µg
$ZnSO_4$ x7H_2O	115µg
$CuCl_2$ x2H_2O	17µg
Na_2MoO_4 x2H_2O	4.84µg
Co $(NO_3)_2$x2H_2O	6.5µg
Na pantothenate	
Thiamine HCl	0.8 mg each
Nicotinamide	
pH	6.8
H_2O	to 1000 ml

For preparation of the media, samples of stock solutions were used.

2.6.2 Competition under normal conditions or stress of salinity and alkalinity

Plant germination, sterilization and cultivation were performed as previously mentioned. The concentration of cultures was adjusted by O.D. to 10^8 cells ml^{-1}. Strains were mixed in a 1:1 ratio for each treatment. Plants were cultivated in the growth chamber under the conditions described before. To study the competition under stress of salinity, sodium chloride was added to the nutrient solution at a final concentration of 0.2% NaCl (34.2 mM). For alkaline stress pH8, the nutrient solution was buffered with 20 mM AMPD (Priefer et al., 2001). To select the competitive strains under stress of high temperature, plants were cultivated in a controlled growth chamber with 15h of light at 35 °C and 9h of darkness at 25 °C, relative humidity of 75% and light intensity 14 K Lux,.

Nodulation parameters were estimated and part of nodulated roots were used for determining the percentages of nodules occupancy by soaking these roots in a buffer containing x-gluc substrate, and counting blue and white nodules. Competitiveness indices were estimated using the equation described by Beattie et al. (1989).

2.6.3 *Gus* staining

Nodulated roots of common bean, inoculated with two strains were soaked in *Gus* staining buffer with 4.35g K_2HPO_4, 3.41g KH_2PO_4, 5 ml of 10% SDS, 2.8 ml of 35% sarcosyl, 1 ml of Triton x-100, 0.37g EDTA, 50 mg of x-gluc (dissolved in DMF), H_2O up to 1000 ml and pH between 7.5-8. The whole root system was incubated with this buffer on a rotary shaker in the dark at 28 $^{\circ}$C for 48 h.

2.7 Effect of nitrogen fertilizer on the symbiotic efficiency of *R. etli* strains to nodulate *Phaseolus vulgaris*

To test the effect of nitrogen fertilizer application on the ability of strains EBRI 2 and EBRI 26 to nodulate and fix nitrogen with *Phaseolus vulgaris*, the nodulation assays were performed in sterilized Leonard jars with vermiculite:perlite (1:1 v/v) and N-free nutrient solution as explained previously. Potassium nitrate (KNO_3) was added to the nutrient solution to give a concentration of 20, 40, 80 and 160 ppm N. Nodule number and fresh weight, shoot fresh weight, percentages of effectiveness and acetylene reduction assay were determined as described before.

2.8 Proteom Analysis

2.8.1 Protein isolation and sample preparation

1- Grow the salt tolerant strains *R. etli* EBRI 26 and *Sinorhizobium meliloti* 2011 in 20E liquid medium amended with 4% NaCl, 2.5% NaCl respectively or without NaCl as a control for 2 days.
2- Cells from 100 ml of bacterial culture were harvested at the mid exponential growth phase by centrifugation at 8000 rpm for 15 min.
3- Cell pellets were re-suspended in 1 ml lysis buffer.
4- Use the French press machine to break the cell walls at 1000 bar.
5- Centrifuge at 20000 rpm for 20 min.
6- Collect the supernatant containing soluble protein.
7- Concentrate the protein samples using a lyophilizer machine and measure the protein concentration according to the method of Bradford (1976).

2.8.2 Preparation of samples for 2D gels electrophoresis

8- Take a volume with 700 µg protein and precipitate the protein with 10% TCA in acetone containing 20 mM DTT and let the samples precipitate overnight at –20 °C.
9- Centrifuge at 20000 rpm and 4 °C for 20 min to pellet the protein and remove the supernatant without pipeting.
10- Wash the pellets with acetone containing 20 mM DTT for 20 min with vortexing to remove traces of TCA.
11- Centrifuge again at the same speed and at 4 °C for 20 min and remove the supernatant carefully.
12- Dissolve the protein pellet in 250 µl of re-hydration buffer.
13- Add 6 µl for each sample from IPG buffer pH 4-7 and traces of bromophenol blue.
14- Vortex them on the platform for 20 min to dissolve the protein in the rehydration buffer.
15- Load the protein samples on the strips (IPG, 13cm, pH 4-7) and let them for rehydration overnight with covering them with paraffin oil to avoid the crystallization of the urea.
16- Take the strips and wash them using distilled water without touching the agarose side and try to absorb the excess of water with absorbent paper.

2.8.3 Running of first and second dimension

1- load the strips (IPG strips 13 or 24 cm long and pH gradient from 4- to 7 from Amersham Biosciences) rehydrated with protein in a 2D machine (the Ettan TM Dalt ∏ system, Amersham Biosciences) and run them first

at 600V for 1h and 6000V for 6h, as isoelectric focusing IEF (first dimension) as described by Rabus et al. (2002).

2- After finishing the first dimension, the strips should be soaked in the equilibration buffer for at least 20 min with slow shaking.
3- Run the samples on 12% polyacrylamide gel electrophoresis PAGE (second dimension) in running buffer at 70 mA for 9-10 h with loading protein standard at both sides (two standard of proteins were used from Biorad, one with high MW from 200 MD to 45 MD and the second with low MW from 97 MD to 14 MD) and sealing the strips with 0.5% agarose before separating the proteins in the second dimension.
4- Transfer the gels very carefully and stain them with CBB commassie brilliant blue dye in methanol modified by Doherty et al. (1998).
5- Check the spots of protein and scan them.
6- Sometimes, it is necessary to do de-staining for the gel in DW to remove the excess of dye.

2.8.4 Preparation of 2D gels

1- To prepare 12% of acrylamide gel, 30 ml of these components 12.5 ml of bei-distilled water, 7.5 ml Tris HCl pH 8.8, 10 ml of 30% acrylamide, 200 µl of APS and 20 µl of TEMED are mixed.
2- Poure this mix immediately after preparation to the tray of the gel and cover the upper layer with 50% propanol.
3- Let the gel polymerize for at least 4 h, note that it is very important to clean the glasses and spacers with ethanol.

2.8.5 Handling of protein spots for analysis by MALDI-TOF

Protein digestion was performed according to the method described by Hellman et al. (1995) as following:

1- Spots of separated proteins were precisely cut out of the gel using the blue pipette tip and put into sterile eppendorf tube.
2- Add 200 µl of washing/de-staining buffer and incubate at 37 $^{\circ}$C for 20 min with shaking.
3- This step can be repeated two or three times until the blue dyes is removed.
4- Remove the washing/de-staining buffer and dry the gel pieces under vacuum for 30 min.
5- Add 10 µl of the digestion solution of trypsin to the gel particles and incubate for 45 min for adsorption, then add more of this solution (5µl), incubate for 1h to ensure that the gel pieces are fully saturated with the digestion solution.

6- When the digestion solution is completely aspirated, the samples should be incubated at 37 °C for 18 h with inverting the eppendorf tube too reduce the evaporation.
7- 15 µl of diffusion solution (10% CH3CN v/v, 10% TFA v/v in HPLC water) was added and the mixture was sonicated at 37 °C for 45 min.
8- Mix 5 µl of the digest mixture with 5 µl of matrix solution, the acidity of matrix solution inactivates the trypsin.
9- Transfer 0.5-1 µl of this mixture to the gold plate of MALDI-TOF machine and allow to dry and analyse the samples with the mass spectrometer.

Solutions

Lysis buffer: (10 mM Tris-HCl pH 7.4, 1 mg ml^{-1} $MgCl_2$, 50 µg ml^{-1} DNAase, 50 µg ml^{-1} RNAase and 50 µg ml^{-1} lysozyme).
Rehydration buffer: (8 M Urea, 2M Thiourea, 4% DTT, 2% CHAPS, 2% IPG buffer and trace of bromophenol blue).
Equilibration buffer: (50 mM Tris-HCl pH 6.8, 6M Urea, 30% v/v glycerol, 2% w/v SDS and amended with 64mM DTT or 135 mM iodoacetamide).
Running buffer: 0.124 M Tris, 0.96 M glycin, 0.05% SDS.
Washing/de-staining buffer: (200 mM NH_4HCO_3, 50% CH_3CN).
Digestion solution: (10% CH_3CN, 40 mM NH_4HCO_3 [pH 8.1], 0.02 µg ml^{-1} sequencing grade trypsin).
Diffusion solution: (10% CH_3CN v/v, 10% TFA v/v in HPLC water)
Matrix solution: (2 mg αCHCA from Sigma company in 170 µl of 70% CH_3CN in 0.1 TFA amended with 0.25 µl of calibration mixture from Applied Biosystems diluted 1:500).

Colloidal commassie staining

Colloidal commassie staining was prepared as described by Doherty et al. (1998) as following:
Staining solution: 0.08% Commassie Brilliant Blue G250 (CBB G250); 1.6% ortho-phosphoric acid; 8% ammonium sulphate and 20% methanol.
Solution A: 2% ortho-phosphoric acid; 10% (w/v) ammonium sulphate. Take 16 ml of ortho-phosphoric and dissolve in 768 ml of distilled water (dw) and dissolve 80g of ammonium sulphate in this solution.
Solution B: prepare 5% of CBB G250 (w/v) by dissolving 1g CBB in 20 ml dw with stirring.
Solution C: Dissolve 16 ml of solution B in solution A to a final volume of 800 ml. Do not filter the solution, the solution can be stored for several weeks at room temperature.

Solution D: Directly before use 200 ml of methanol should be added to 800 ml of solution C, with final a solution of 1000 ml. The gels should be stained in solution D.

2.8.6 Labelling of protein with Cyanine dyes

Samples of protein were labelled with dyes according to the method published by Gade et al. (2003) and outlined below:

1- Take a volume with a bout 50 µg of total soluble protein and add 20 µl of rehydration buffer, but the IPG buffer and DTT should be omitted to avoid to react with NHS esters of the cyanine dye.
2- Adjust the pH to 8.5 using 50 mM NaOH for a good labelling with the dyes.
3- Prepare stock solution of labelling dye by dissolving 1 ng fluor dye μl^{-1} of DMF, and dilute this solution to 400 pmol μl^{-1} of DMF (working solution).
4- For labelling the samples, 50 µg protein must be mixed with 400 pmol of dye, vortex them and incubate them on ice for 30 min in the dark.
5- Stop the reaction by adding 1 µl of 10 mM lysine, then vortex again and incubate on ice for 10 min.
6- Added rehydration buffer with DTT and IPG buffer to a volume of 500 µl. The samples are now ready for the first dimension (IEF) and the second dimension as explained previously.
7- To detect the labelled spots of protein, gels are scanned directly using the Typhoon Scanner 9400 (Amersham Bioscineces). Results from 2D are analysed using DeCyder software package (version 4.0; Amercham Biosciences) according to the method explained by Gade et al. (2003).

3 Results

3.1 Efficiency and host specificity of rhizobial strains

Twelve rhizobial isolates were recovered from *Phaseolus vulgaris* nodules sampled at two regions in Egypt Table 1. Seven isolates were from Ashmun area in the middle of the Delta-Nile valley and five were from the Isma'ilya desert area where common bean are cultivated repeatedly. Nodulation phenotypes for these isolates with cultivar Saxa are shown in Table 6. The best strain for nodule formation was EBRI 3 (129 nodules plant^{-1}) followed by EBRI 29 and CIAT 899. Nodule fresh weight was also highest with strain EBRI 3, followed by EBRI 29 and EBRI 21. Plants inoculated by strains EBRI 21, 24, 3 and CIAT 899 produced the highest shoot biomass compared to control plants. It is remarkable, that the weight of nodules formed by strains EBRI 3, 27 and 29 was higher than the weight of the root system after detaching the nodules (Table 6). Isolate EBRI 21 gave the highest specific ARA activity with 13.7 n mol C_2H_4 h^{-1} mg $^{-1}$ nodule followed by strains EBRI 2 and EBRI 26. Strains EBRI 6, 20, 23 and 25 failed to re-nodulate the host *Phaseolus vulgaris*.

Based on these results we selected the strains EBRI 2, 21 and 26 and the reference strain *R. tropici* CIAT899 to study the N_2 fixation capacity of these isolates with three *Phaseolus* cultivars, Giza 6, Canoca and Saxa, 30 days after inoculation (Table 7). With the Egyptian cultivar Giza 6, the strains from Egypt formed the largest number of nodules and the highest nodule biomass. Strains CIAT 899 and EBRI 26 were the most effective ones for nitrogen fixation, with 16.9 and 13.2 n mol C_2H_4 h^{1} mg^{-1} nodule. With the cultivar Canoca from Columbia, the South American strain CIAT 899 gave the highest nodule biomass compared to the three strains from Egypt. With the European cultivar Saxa the best strain for nodule biomass was EBRI 26 while Strain EBRI 21 gave

the highest shoot biomass. From the three strains tested in this series, only strain EBRI 2 produced consistently a nodule/root biomass ratio under 1, whereas with the other three strains this ratio was more than 1 (Table 7).

Table 6. Nodulation data of new isolates of bean rhizobia nodulating *Phaseolus vulgaris cv*. Saxa, 21 days after planting.

Treatments with strains	**No. of nodules plant^{-1}**	**FW of nodules mg plant^{-1}**	**FW of shoots g plant^{-1}**	**FW of roots mg plant^{-1}**	**n mol C_2H_4 h^{-1} mg^{-1} nodules**	**Ratio of nodule to root FW**
No inoculation	0.0	0.00	1.8	1250	0.00	-
CIAT 899	71	578	2.4	830	6.5	0.70
EBRI 2	67	510	2.1	1140	8.9	0.45
EBRI 3	129	907	2.4	870	3.7	1.04
EBRI 6	0.0	0.0	1.9	780	0.0	-
EBRI 20	0.0	0.0	1.8	260	0.0	-
EBRI 21	73	695	2.6	1160	13.7	0.56
EBRI 23	0.0	0.0	1.7	340	0.0	-
EBRI 24	60	670	2.5	690	7.5	0.97
EBRI 25	0.0	0.0	1.9	900	0.0	-
EBRI 26	62	507	2.4	930	7.9	0.55
EBRI 27	67	561	2.2	350	6.2	1.60
EBRI 29	79	715	2.2	290	6.4	2.46
EBRI 32	52	600	2.0	650	Nd	0.92
L.S.D 0.05	**27.8**	**281**	**N.S**	**240**	**3.82**	**-**

Results are taken from three replicates for each parameter, Ns: non significant result. Nd: not determined, FW: fresh weight.

Table 7. Nodulation data of selected isolates of bean rhizobia with different host cultivars 30 days after planting.

Treatments with strains	**No. of nodules plant^{-1}**	**FW of nodules mg plant^{-1}**	**FW of shoots g plant^{-1}**	**FW of roots mg plant^{-1}**	**n mol C_2H_4 h^{-1} mg^{-1} nodules**	**Ratio of nodule to root FW**
Plant cultivar Giza 6						
No inoculation	0.0	0.0	5.1	2050	0.0	-
CIAT 899	132	812	9.8	390	16.7	2.1
EBRI 2	192	1692	14.4	2300	7.1	0.73
EBRI 21	170	991	12.3	890	9.5	1.11
EBRI 26	165	896	10.8	800	13.2	1.12
Plant cultivar Canoca						
No inoculation	0.0	0.0	2.3	1390	0.0	-
CIAT 899	153	1289	10.1	800	9.4	1.61
EBRI 2	91	838	8.3	1310	14.3	0.63
EBRI 21	161	772	9.0	490	7.6	1.58
EBRI 26	203	948	8.3	790	12.7	1.20
Plant cultivar Saxa						
No inoculation	0.0	0.0	2.5	1600	0.0	-
CIAT 899	113	870	8.3	400	9.4	2.2
EBRI 2	116	1005	9.8	1600	14.3	0.63
EBRI 21	141	949	10.1	700	7.6	1.44
EBRI 26	199	1051	8.69	900	12.7	1.16
L. S. D. 0.05	**62.91**	**460**	**3.57**	**530**	**5.02**	**-**

FW: fresh weight.

The un-inoculated plants for all the three cultivars suffered from nitrogen deficiency in the nutrient solution, they have the yellow colour (cholorosis) compared to the green colour of inoculated plants. After 30 days, the cultivar Saxa was the only cultivar which entered in to the flowering stage Figures 3a, b, c.

Figure 3a. Cultivar Giza 6 inoculated with CIAT 899 and Egyptian bean rhizobial isolates EBR2, 21 and 26.

Figure 3b. Cultivar Canoca inoculated with CIAT 899 and Egyptian bean rhizobial isolates EBRI 2, 21 and 26.

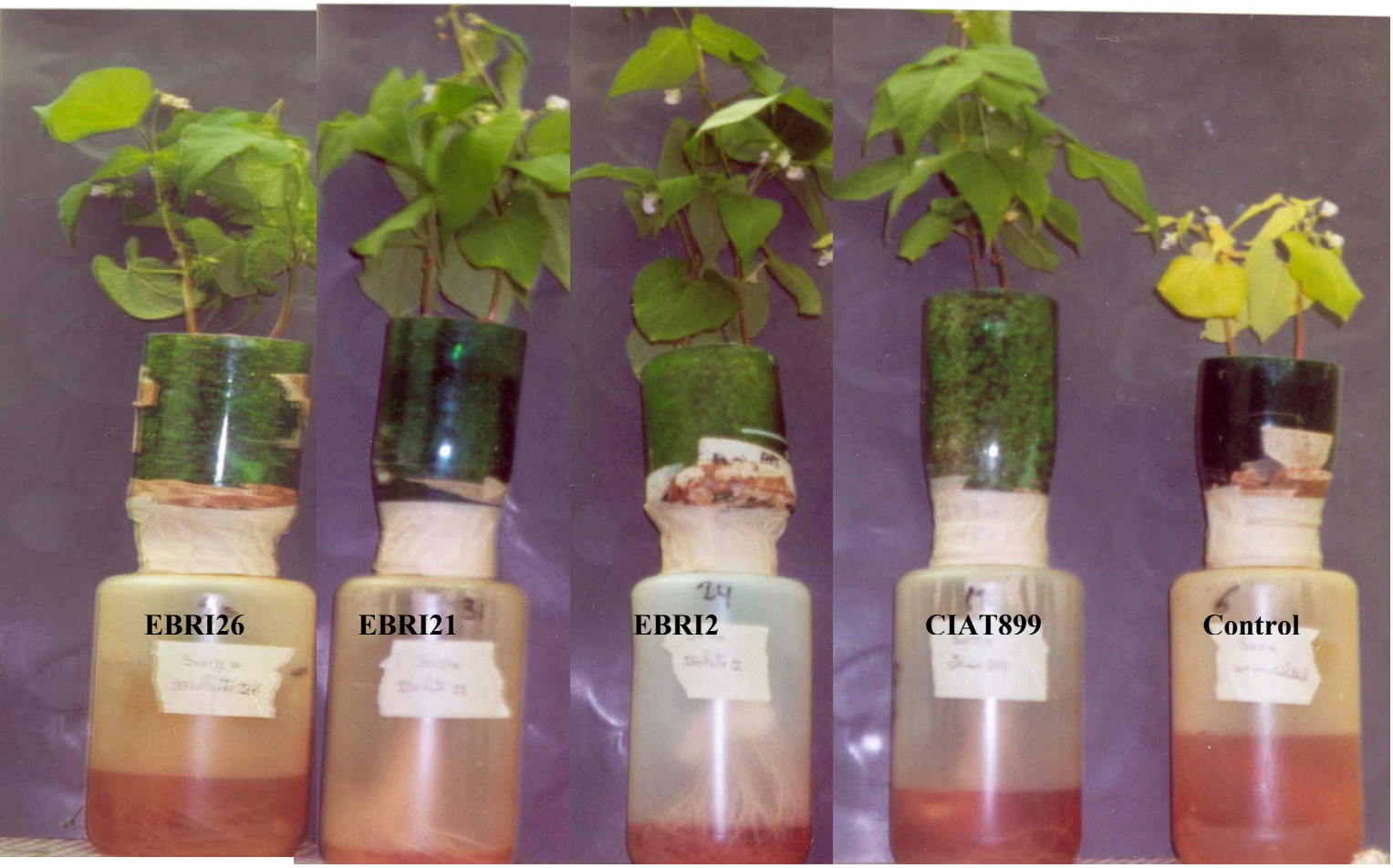


Figure 3c. Cultivar Saxa inoculated with CIAT 899 and Egyptian bean rhizobial isolates EBRI 2, 21 and 26.

3.2 Genotypic characterization of bean rhizobial isolates from Egyptian soils using different finger printing methods

DNA was extracted from the strains using the cetyltrimethyl-ammonium bromide method Figure (4). The characteristics of DNA from the selected strains did show, that the samples were pure and free from contaminating proteins, but some samples had RNA inpurities. DNA samples were not degraded. The concentration of DNA varied between 85-616 µg ml $^{-1}$.

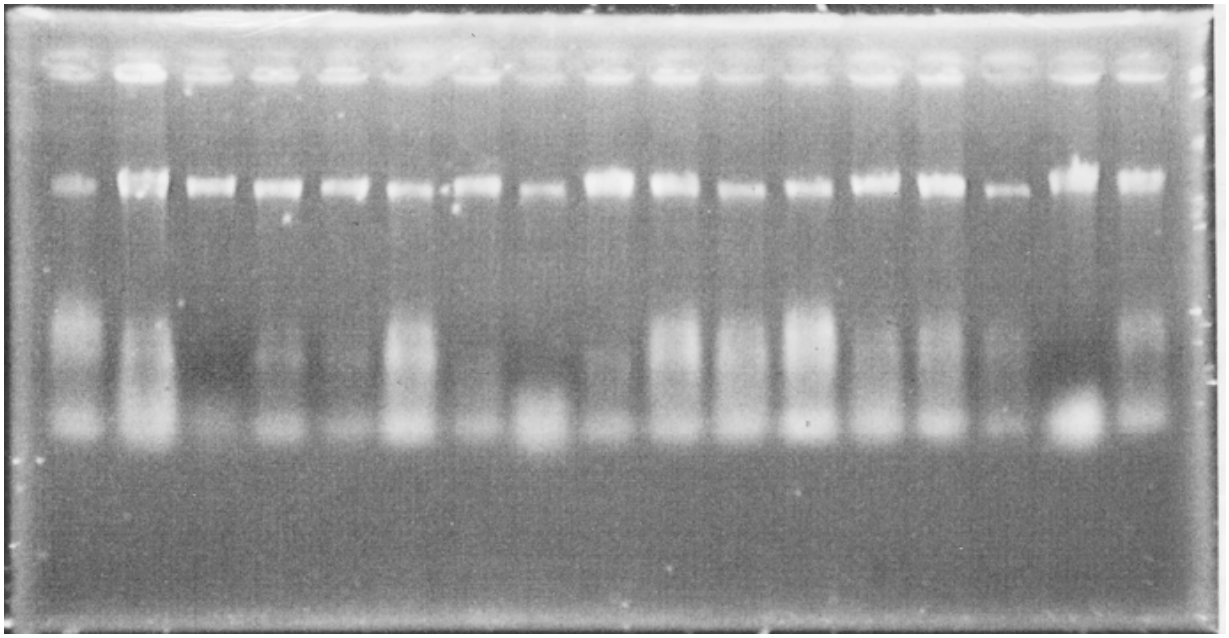


Figure 4. DNA isolation from the isolates and standard strains using the method of cetyl-trimethyl-amoniumbromide (CTAB).

REP-PCR fingerprints are highly discriminatory, allowing the identification and discrimination of strain within a bacterial species. This technique was used for primary grouping of 12 isolates of bean rhizobia nodulating *Phaseolus vulgaris*. Primers Rep1 and Rep2 (Versalovic et al., 1994) were used to amplify repetitive DNA sequences present in multiple copies in the genome of most gram negative bacteria like rhizobial isolates. Results in Figure 5 revealed a high degree of diversity among the strains. The isolates formed four groups. Strains EBRI 26, 27, 29 fell within one group. Strains EBRI 2 and EBRI 3 were closest together.

Strains EBRI 6 and EBRI 25 were identical. The last group contained strains EBRI 20, 21, 23 and 24. No dominant strain was found in the two locations of origin in Egypt.

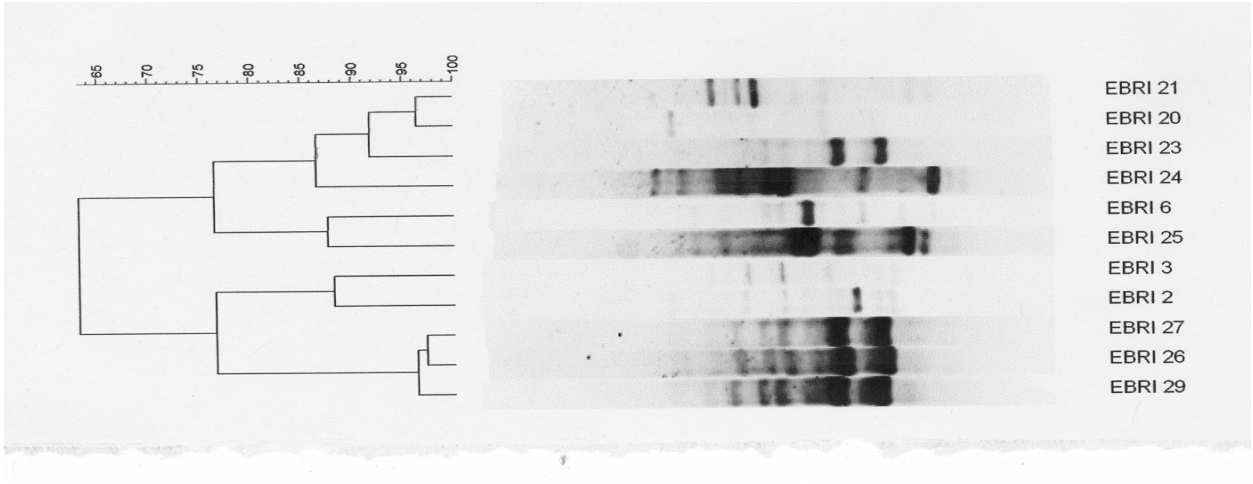


Figure 5. Pearson correlation of REP-PCR for rhizobial isolates from Egypt, at similarity level 65%.

3.2.1 Typing of bean rhizobial isolates by 16S rDNA-PCR / RFLP analysis

Primers fD1 and rD1 (Weisburg et al., 1991) were used to amplify the 16S rDNA region (rrs) from the 12 isolates, in addition to standard strains. All of them gave products of a bout 1500 bp (Figure 6).

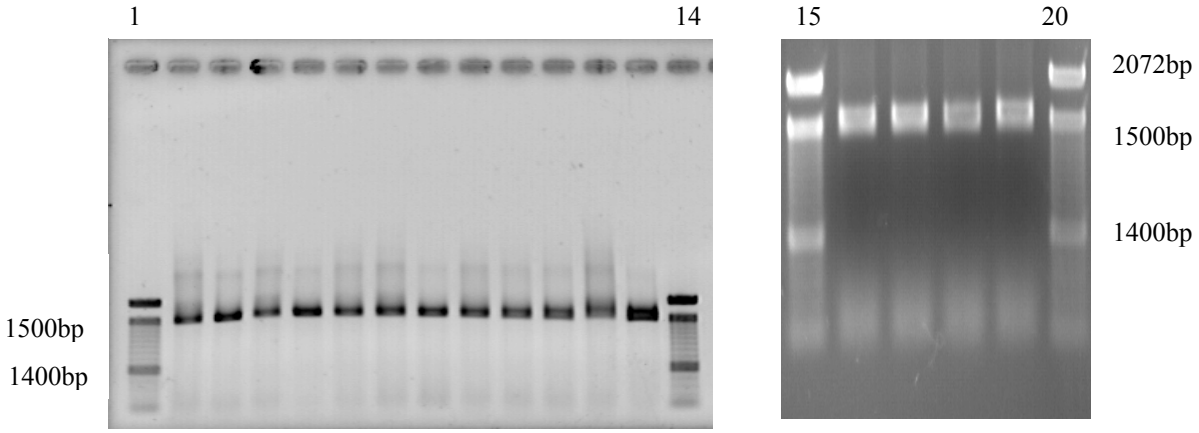


Figure 6. Amplification of 16S rDNA fragments for all rhizobial isolates from Egyptian soils and standard strains, lanes 1, 14, 15, 20 are 100 bp ladders, from left to right: *R. tropici* CIAT899, EBRI 2, 3, 6, 20, 21, 23, 24, 25, 26, 27, 29, *R. gallicum* R602sp, *R. giardinii* H152, *R. etli* CFN42 and EBRI32

Products from amplification of rrs were subjected to RFLP analysis using digestion with the four restriction enzymes *Hha1, Dde1, Msp1* and *Sau3a1*. The expected fragments, their sizes and the sum of their lengths are listed in Table A4. Restriction patterns of rrs using *HhaI* (Figure 7) gave four major genotype groups. The largest group contained the six strains, EBRI 2, 3, 21, 26, 27 and 32 which were closest to the standard strains *R. etli* CFN42, *R. gallicum* R602sp and *R. giardinii* H152. The second group included strains EBRI 6, 20, 23 and 25. Strains EBRI 24 and EBRI 29 had a unique pattern of fragments similar to *R. tropici* CIAT 899.

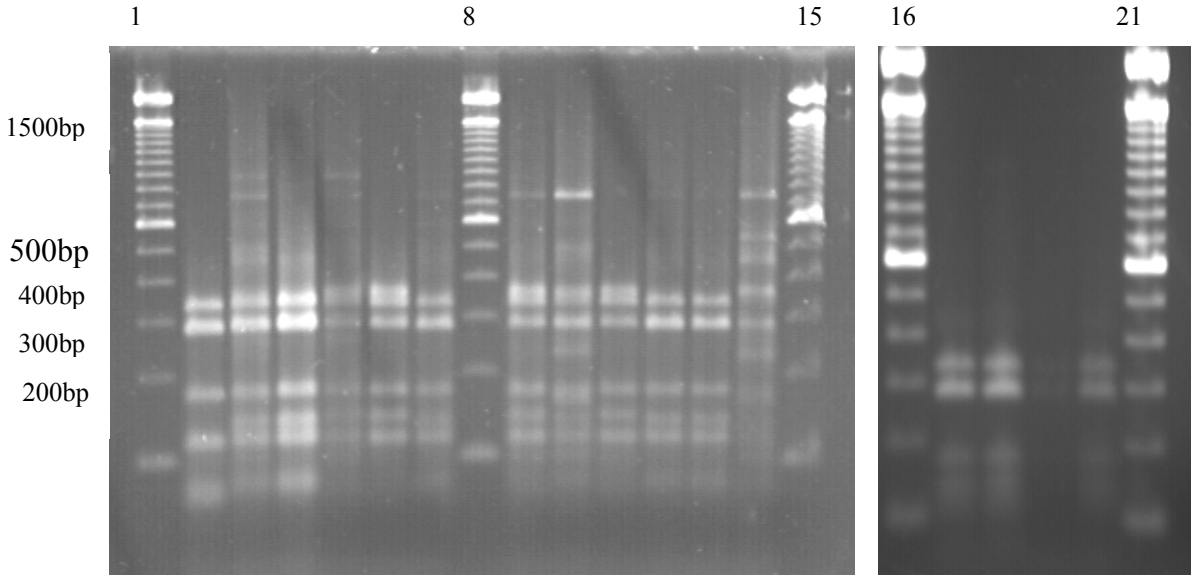


Figure 7. Restriction analysis of amplified fragment of 16S rDNA with enzyme *HhaI,* lanes 1, 8, 15, 16, 21 are 100 bp ladders, from left to right: *R. tropici* CIAT899, EBRI 2, 3, 6, 20, 21, 23, 24, 25, 26, 27, 29 , *R. gallicum* R602sp, *R. giardinii* H152, *R. etli* CFN42 and EBRI32.

Fragments of DNA obtained by cutting the product of rrs with *DdeI* (Figure 8) gave two main groups. In the first group, strains EBRI 2, 3, 21, 24, 26, 27, 29, and 32 shared the DNA profile with strains of *R. etli* CFN42, *R. gallicum* R602sp and *R. tropici* CIAT 899. The second group included the isolates EBRI 6, 20, 23, 25 and strain *R. giardinii* H152.

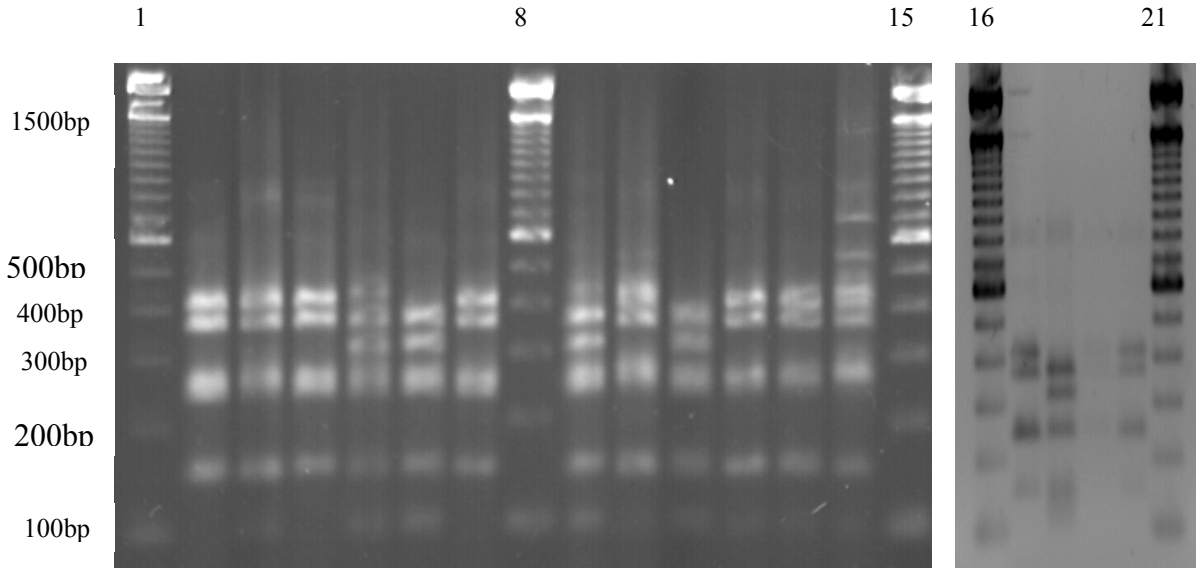


Figure 8. Restriction analysis of the amplified fragment of 16S rDNA digested with enzyme *DdeI,* lanes 1, 8, 15, 16, 21 are 100 bp ladders, from left to right: *R. tropici* CIAT 899, EBRI 2, 3, 6, 20, 21, 23, 24, 25, 26, 27, 29, *R. gallicum* R602sp*, R. giardinii* H152, *R. etli* CFN42 and EBRI 32.

The digestion of rrs fragment with enzyme *MspI* (Figure 9) generated the same DNA fingerprinting types as produced with *HhaI* enzyme except that strains *R. tropici* CIAT 899 and *R. giardinii* H152 fell within one group.

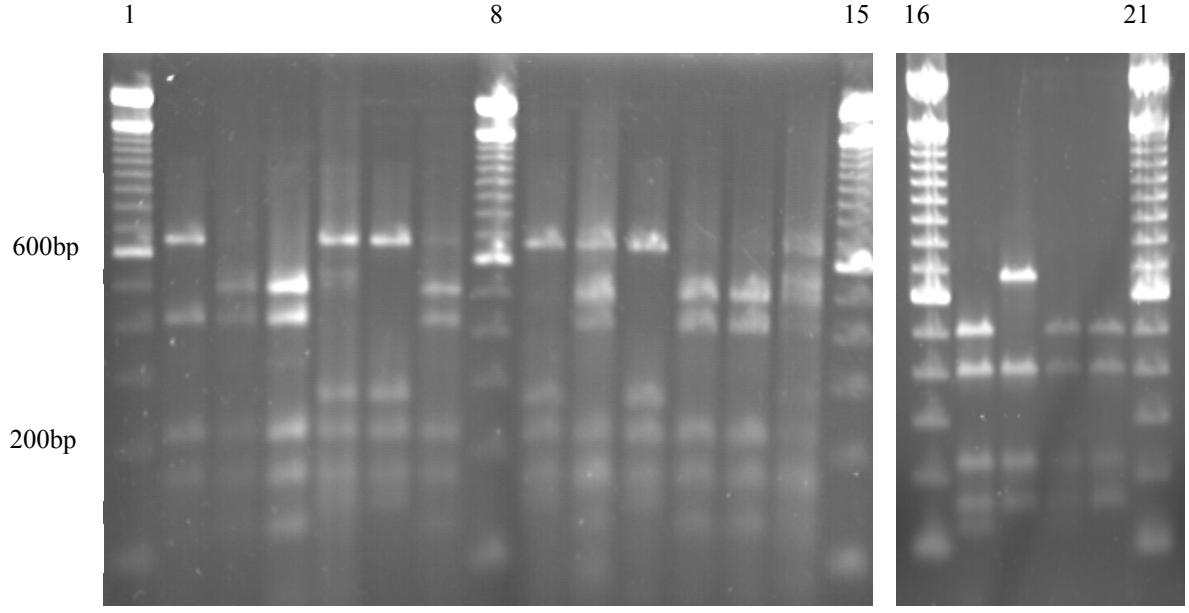


Figure 9. Restriction analysis of the amplified fragment of 16S rDNA digested with enzyme *MspI,* lanes 1, 8, 15, 16, 21 are 100 bp ladders, from left to right: *R. tropici* CIAT 899, EBRI 2, 3, 6, 20, 21, 23, 24, 25, 26, 27, 29, *R. gallicum* R602sp, *R. giardinii* H152, *R. etli* CFN42 and EBRI 32.

Restriction analysis of rrs fragment with *Sau3AI* (Figure 10) created six genotypes. The Egyptian strains EBRI 2, 3, 21, 26 and 27 with the standard strain *R. etli* CFN 42 were included in one genotype. The second genotype were the isolates EBRI 6, 20, 23 and 25. Strains EBRI 24 and EBRI 29 had the same genotype. Strain EBRI 32 shared the same genotype with the strain *R. gallicum* R602sp. *R. tropici* CIAT 899 and *R. giardinii* formed two separate genotypes.

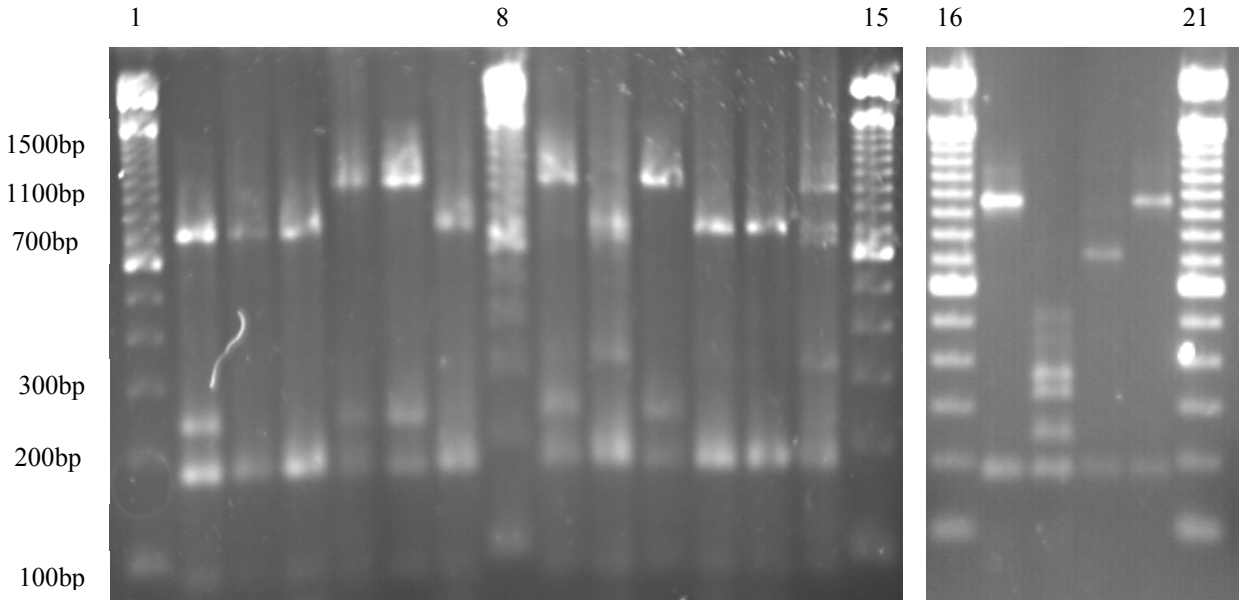
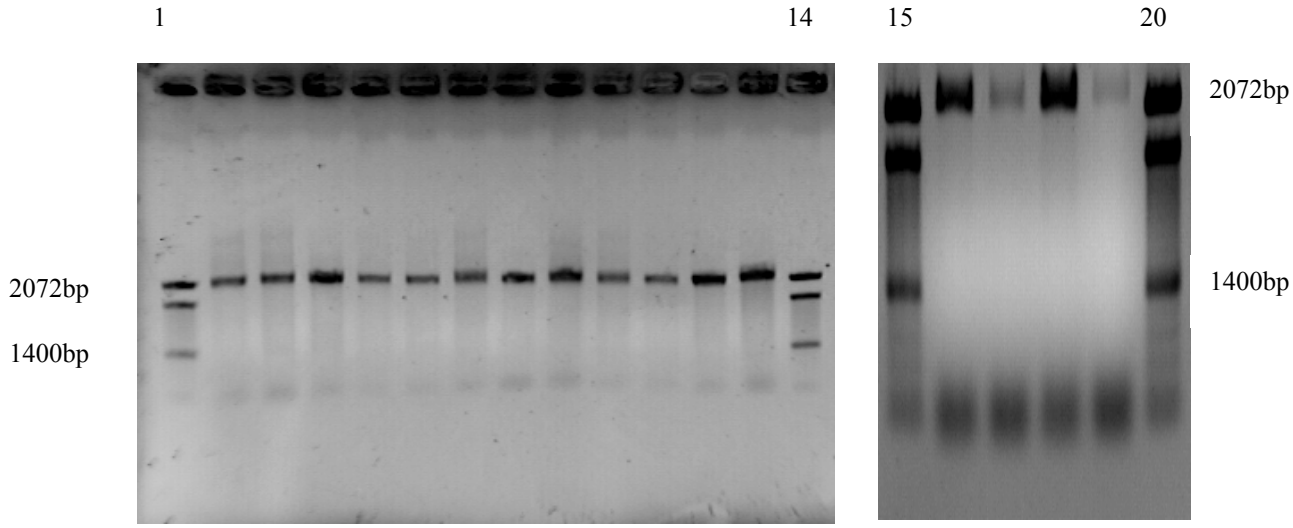


Figure 10. Restriction analysis of 16S rDNA fragments with Sau3a*I*, lanes 1, 8, 15, 16, 21 are 100 bp ladders, from left to right: *R. tropici* CIAT 899, EBRI 2, 3, 6, 20, 21, 23, 24, 25, 26, 27, 29, *R. gallicum* R602sp, *R. giardinii* H152, *R. etli* CFN42 and EBRI32.

3.2.2 Typing of Bean rhizobial isolates by 23S rDNA-PCR/ RFLP analysis

The 23S rDNA amplicon (rrl) for the twelve isolates plus the standard strains was amplified with the primers P3 and P4. All examined strains generated amplification product of about 2100 bp (figure 11). The amplified fragments were digested with the three restriction enzymes *HinfI*, *Sau3aI* and *MspI*. The genetic diversity of the isolates and standard strains was higher than that obtained from 16S rDNA.



Restriction analysis of rrl with enzyme *HinfI* (Figure 12) revealed six genotypes. The first genotype included strains EBRI 2, 3, 21, 24, 26, 27 and *R. tropici* CIAT 899. The second group contained the four strains EBRI 6, 20, 23 and 25. Strain EBRI 32 shared the genotype with *R. gallicum* R602sp. Strains EBRI 29, *R. etli* CFN42 and *R. giardinii* H152 formed separate genotypes.

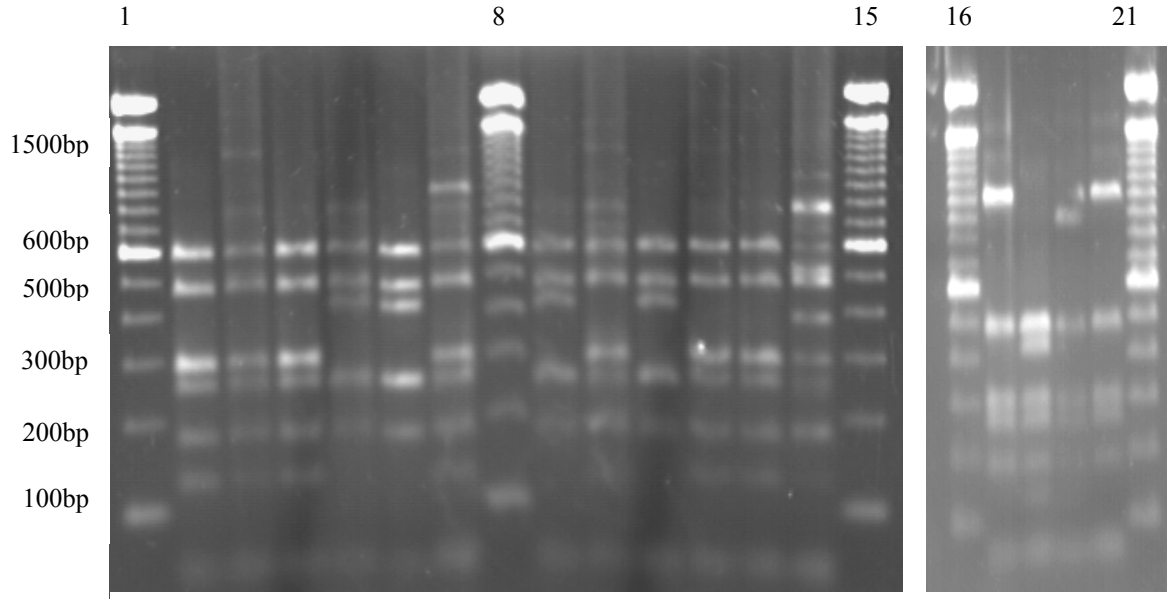


Figure 12. Restriction analysis of the amplified fragment of 23S rDNA cut with Enzyme *HinfI*, lanes 1, 8, 15, 16 , 21 are 100 bp ladders, from left to right: *R. tropici* CIAT899, EBRI 2, 3, 6, 20, 21, 23, 24, 25, 26, 27, 29, *R. gallicum* R602sp, *R. giardinii* H152, *R. etli* CFN42 and EBRI32.

The grouping of strains based on the restriction patterns from cutting the amplified fragment of rrl with enzyme *Sau3aI* **(**Figure 13), gave the same classification as with enzyme *HinfI* except strain *R. tropici* CIAT 899 formed a separate genotype.

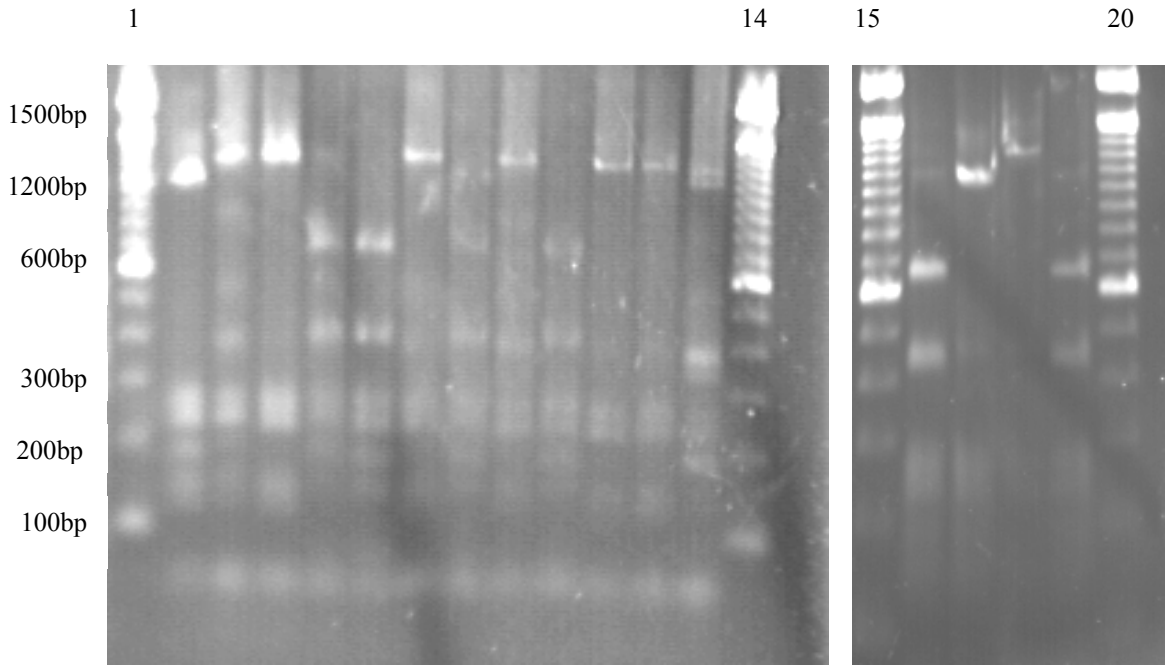


Figure 13. Restriction analysis of amplified fragment of 23S rDNA digested with *Sau3a1,*lanes 1, 14, 15, 20 are 100 bp ladders, from left to right: *R. tropici* CIAT899, EBRI 2, 3, 6, 20, 21, 23, 24, 25, 26, 27, 29, *R. gallicum* R602sp, *R. giardinii* H152, *R. etli* CFN42 and EBRI32.

Also fragment analysis produced with enzyme *MspI* (figure 14) was similar with that obtained from *HinfI* except, that strain *R. tropici* CIAT 899 was a separate genotype and *R. etli* CFN 42 shared the DNA fingerprint with *R. gallicum* R602sp and EBRI 32. The expected fragments, their sizes and the sum of their lengths of amplified fragment of 23S rDNA are listed in Table A5.

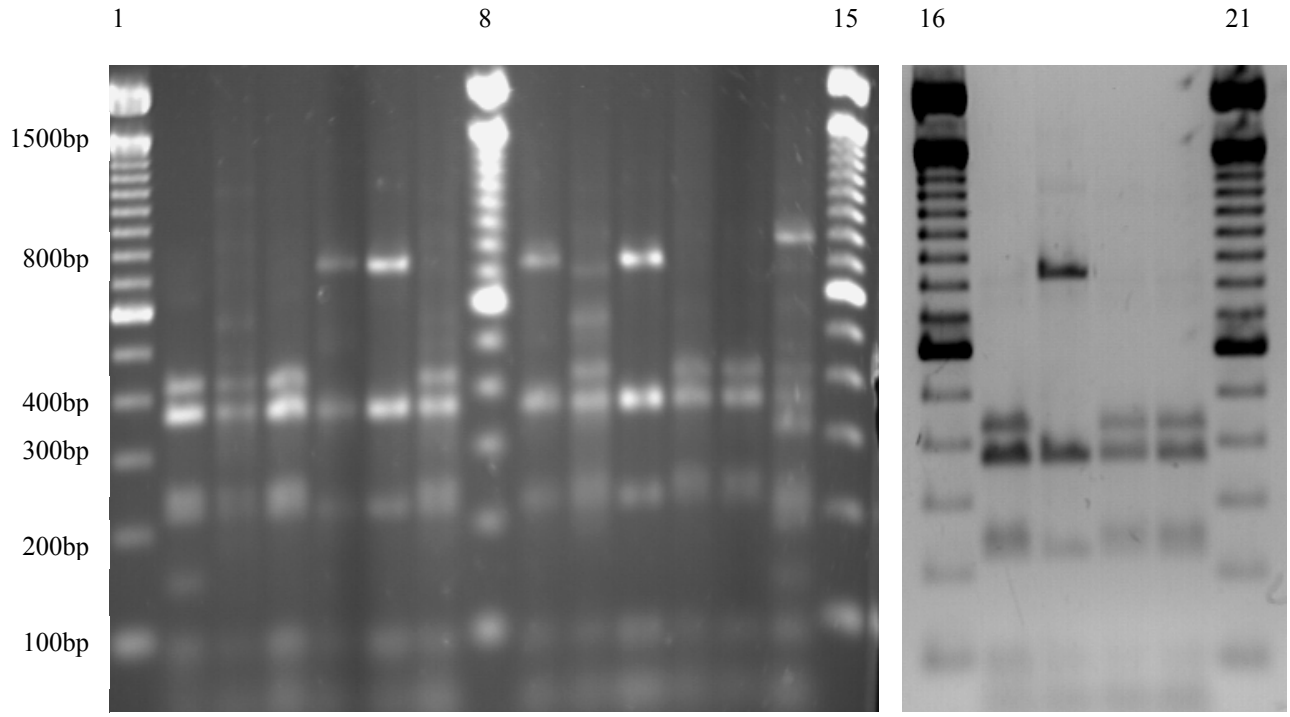


Figure 14. Restriction analysis of the amplified fragment of 23S rDNA digested with Enzyme *MspI,* lanes 1, 8, 15, 16, 21 are 100 bp ladders, from left to right: *R. tropici* CIAT899, EBRI 2, 3, 6, 20, 21, 23, 24, 25, 26, 27, 29, *R. gallicum* R602sp, *R. giardinii* H152, *R. etli* CFN42 and EBRI 32.

3.2.3 Plasmid profile analysis for rhizobial strains

Plasmid patterns were identified with the twelve rhizobial isolates and four standard type strains. Plasmid numbers and their sizes are listed in Table A6. Plasmid numbers ranged from 2 to 6 plasmids and the size was from 40 kb to 650 kb. The plasmid profile analysis gave more diverse groupings among the strains than 16S rDNA and 23S rDNA analysis. While three genotypes were produced from the ARDRA technique, twelve genotypes were generated from plasmid analysis. Results in Figures 15a, b, c and d show the plasmid number for each strain and the expected size compared with the plasmid DNA content from *R. etli* CFN42. Eight strains (*R. etli* CFN 42, EBRI 2, EBRI 3, EBRI 21, EBRI 24, EBRI 26, EBRI 27 and EBRI 29) contained a plasmid with about 650 kb, five strains (*R. etli* CFN 42, EBRI 2, EBRI 3, EBRI 24, EBRI 26 and EBRI 27) harboured a plasmid with about 510 kb. Seven strains (*R. etli* CFN 42,

EBRI 2, EBRI 3, EBRI 24, EBRI 26, EBRI 27 and EBRI 29) had a plasmid DNA with about 390 kb. The four strains EBRI 6, 20, 23 and 25 which failed to re-nodulate the host plant and similar based on the ARDRA method, had different plasmid profiles compared to rhizobial strains. Strain EBRI 24 and EBRI 27 were identical. Strain EBRI 32 shared the plasmid profile with *R. gallicum* R602sp and *R. tropici* CIAT899 not considering the mega plasmids.

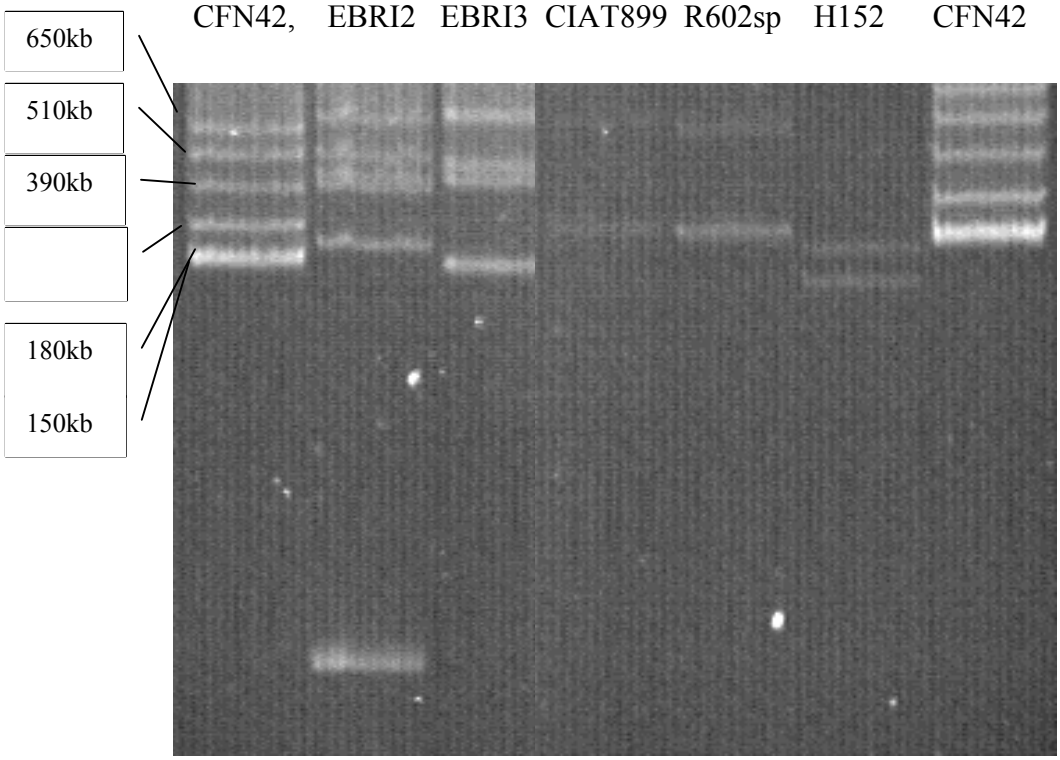


Figure 15a. Plasmid profile analysis of *R. etli* CFN 42, EBRI2, EBRI3, *R. tropici* CIAT899, *R. gallicum* R602sp, *R. giardini* H152 and *R. etli* CFN 42.

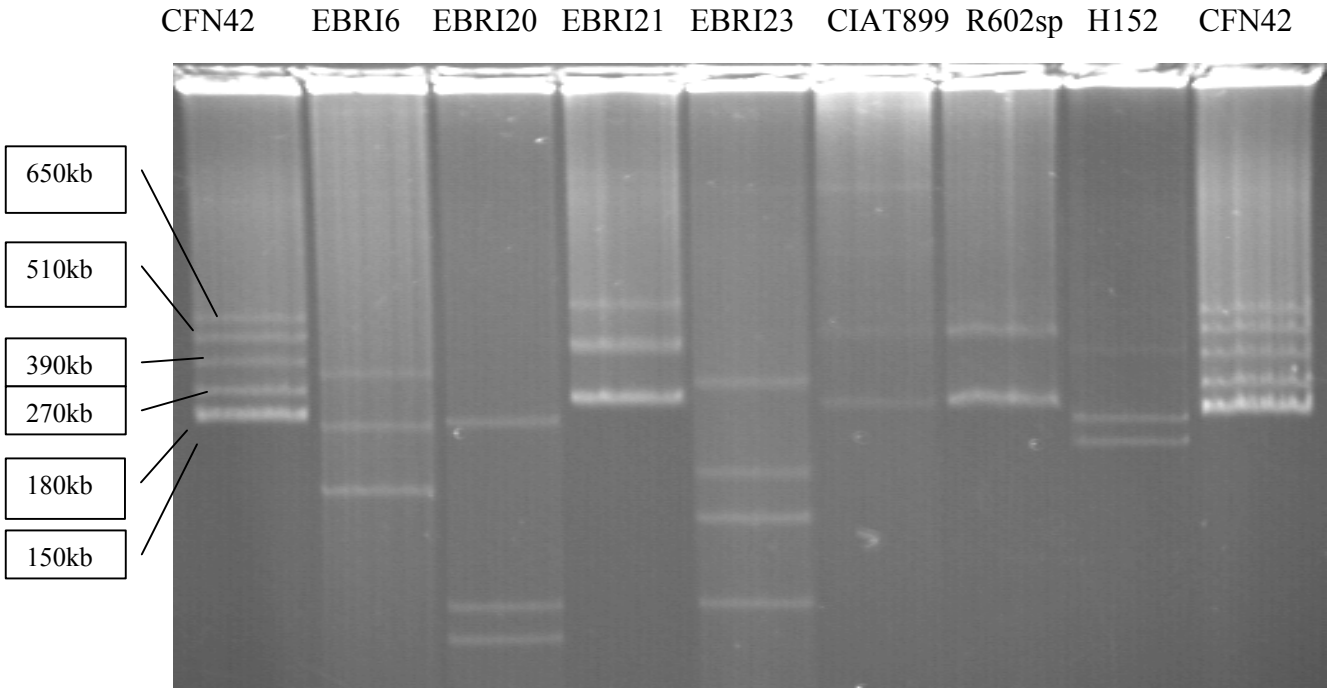


Figure 15b .Plasmid profile analysis of strains *R. etli* CFN42, EBRI6, EBRI20, EBRI21, EBRI23, *R. tropici* CIAT 899, *R. gallicum* R602sp, *R. giardini* H152 and *R. etli CFN42*.

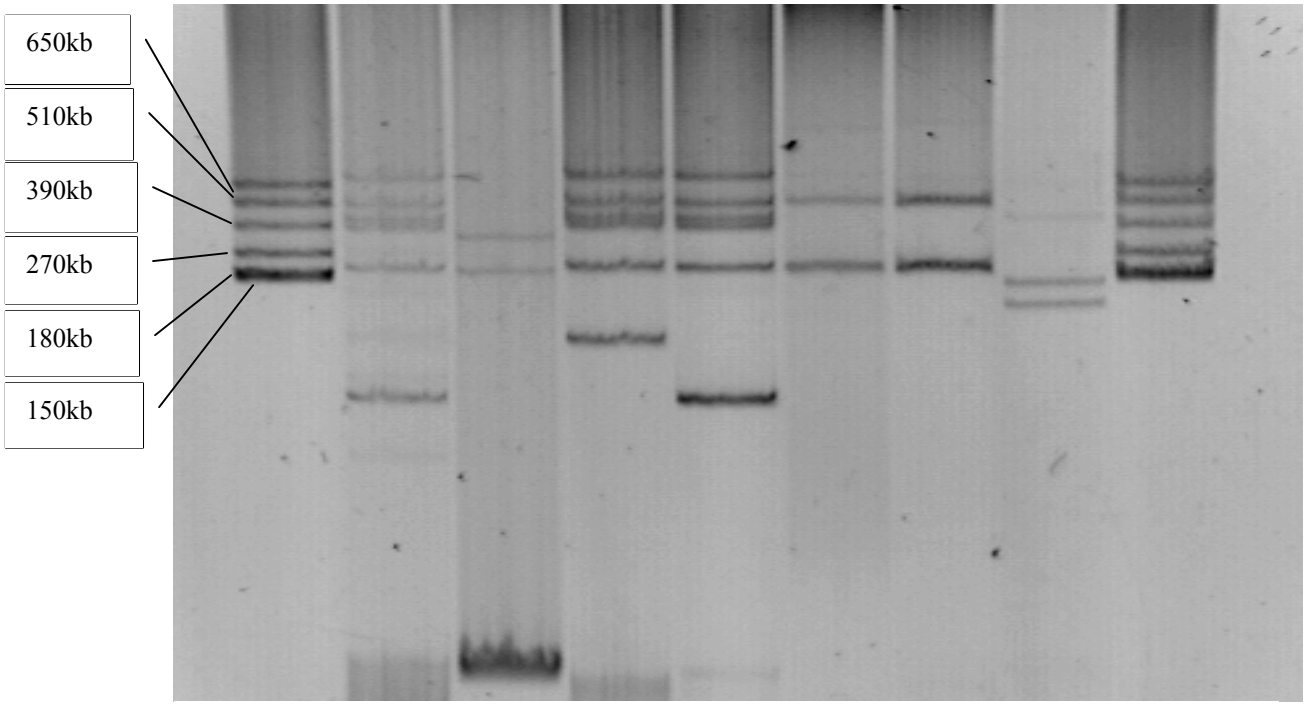


Figure 15c. Plasmid profile analysis of strains *R. etli* CFN42, EBRI24, EBRI25, EBRI26, EBRI27, *R. tropici* CIAT 899, *R. gallicum* R602sp, *R. giardini* and *R. etli* CFN42

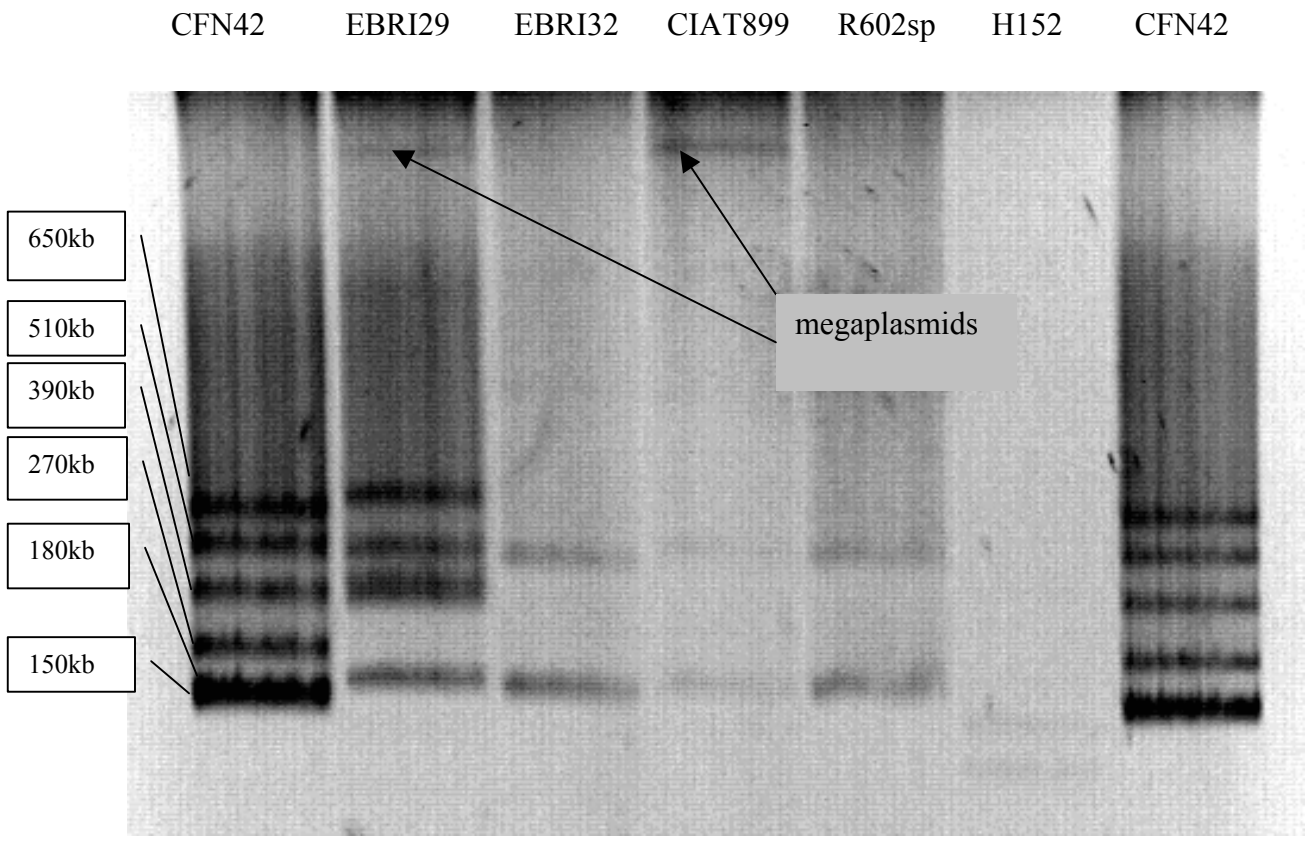


Figure 15d. Plasmid profile analysis of strains *R. etli* CFN42, EBRI29, EBRI32, *R. tropici* CIAT 899, *R. gallicum* R602sp, *R. giardinii* H152 and *R. etli* CFN42.

Only two strains which contained mega plasmids, strains *R. tropici* CIAT 899 and *R. etli* EBRI 29 had one mega plasmid, but the mega plasmid not detected by *R. gallicum* R602sp and EBRI 32 (Figure 15d).

3.2.4 Typing of bean rhizobial isolates by ITS rDNA-PCR/ RFLP analysis

To complete the entire ribosomal DNA analysis the primers FGPS and FGPL were used to amplify the ITS region. All strains gave amplified fragment of about 1420 bp except strain CIAT 899 yielding a product >1420 bp. Strains EBRI 20, 25 and 29 gave two amplified fragments, therefore the generated fragments were not subjected to RFLP analysis (data not shown).

3.2.5 16S rDNA sequencing and identification of rhizobial isolates

Six bean symbionts (EBRI 2, EBRI 3, EBRI 21, EBRI 26, EBRI 27 and EBRI 32) and two non symbiotic strains (EBRI 20 and EBRI 25) were selected for the sequence analysis of the 16S rDNA. The alignment sequence for the strains are given in Figure A1. Results in Table 8 summarize the information and the identification of strains based on the sequence analysis, together with the sequence of some standard strains from the databank. Strains EBRI 2, 3, 21, 26 and 27 were identified as *R. etli*, with identities of 98%, 99.72%, 98.8% , 99.21% and 99.57% to strains *R. etli* TAL182, CFN42, strain C, strain C and strain P respectively. These strains are able to nodulate the common beans. Strains EBRI 20 and EBRI 25 had similarity of 99.8% and 99.9% with *Agrobacterium tumefaciens*. These strains failed to re-nodulate the host of *Phaseolus vulgaris*. Based on these results, they are identified as *Agrobacterium tumefaciens*. Strain EBRI 32 was the only strain identified as *R. gallicum*, because it had a 97% similarity with *R. gallicum* strains. Also, this strain has the ability to form nodules on the root of legume tree (*Leucaena leucocephala*) in addition to *Phaseolus vulagris* as reported previously with *R. gallicum* (Figure 16).

Figure 16. Strain EBRI 32 nodulating a. *Phaseolus vulgaris* and b. *Leuceana leucocephala*.

Table 8. Sequencing of 16S rDNA of Egyptian bean rhizobial isolates compared with sequences from Databank.

Isolate-code	Sequence length	Closest 16 rDNA sequence	Accessions number	Identity %	Identification	Nodulation of host	
						A	B
EBRI 2	1393 nt	*R. etli*	RE47303	98.0%	*R. etli*	+	-
		R. etli TAL 182	RE28939	98.0%			
EBRI 3	1407 nt	*R. etli* CFN42	RE28916	99.7 %	*R. etli*	+	-
		R. etli TAL 182	RE28939	99.6 %			
		R. etli strain P	AY117632	99.7%			
EBRI 20	1406 nt	*A. tumefaciens*	ATU389902	99.8 %	*Agrobacterium tumefaciens*	-	-
		Rhizobium sp					
EBRI 21	1420 nt	*R. etli* Strain C	AF313904	98.8 %	*R. etli*	+	-
		R. etli	RE47303	97.8 %			
EBRI 25	1424 nt	*A. tumefaciens*	ATU389897	99.9 %	*Agrobacterium tumefaciens*	-	-
		Rhizobium sp SPGH 2001	AY029336	99.3 %			
EBRI 26	1406 nt	*R. etli* Strain C	AF 313904	99.2 %	*R. etli*	+	-
		R. etli TAL 182	RE 28939	99.0 %			
EBRI 27	1399 nt	*R. etli* Strain C	AF 313904	99.6 %	*R. etli*	+	-
		R. etli Strain P	AY 117632	99.6 %			
EBRI 32	1356 nt	*R. gallicum*	AF417561	97.0 %	*R. gallicum*	+	+
		R. gallicum	AF417557	97.0 %			

EBRI Egyptian Bean Rhizobial Isolates. A *Phaseolus vulgaris*, B. *Leuceana leucocephala.*

For further proof that the non symbiotic strains (EBRI 6, 20, 23 and 25) are *Agrobacterium tumefaciens*, two additional experiments were done. The first trial was to include the standard strain of *Agrobacterium tumefaciens* 30150 from DSM in the RFLP analysis of 16S rDNA and 23S rDNA. The primers fD1 and rD1 were used to amplify the gene codes for 16S rDNA, and primers P3 and P4 were used to amplify the gene coding for 23S rDNA as described previously. The strain of *Agrobacterium tumefaciens* gave amplified product of about 1500 bp with 16S rDNA and 2100 bp with 23S rDNA as obtained with the other strains before, (data not shown). The amplified fragments of 16S and 23S rDNA were subjected to restriction analysis with enzymes *Hha1*, *Dde1*, *Msp1* and *Sau3a1* for rrs, and *HinfI*, *Sau3aI* and *MspI* for rrl. Results in Figures 17a, b, and 18a, b indicate that the four non bean symbionts strains are identical with the standard strain of *Agrobacterium tumefaciens* 30150.

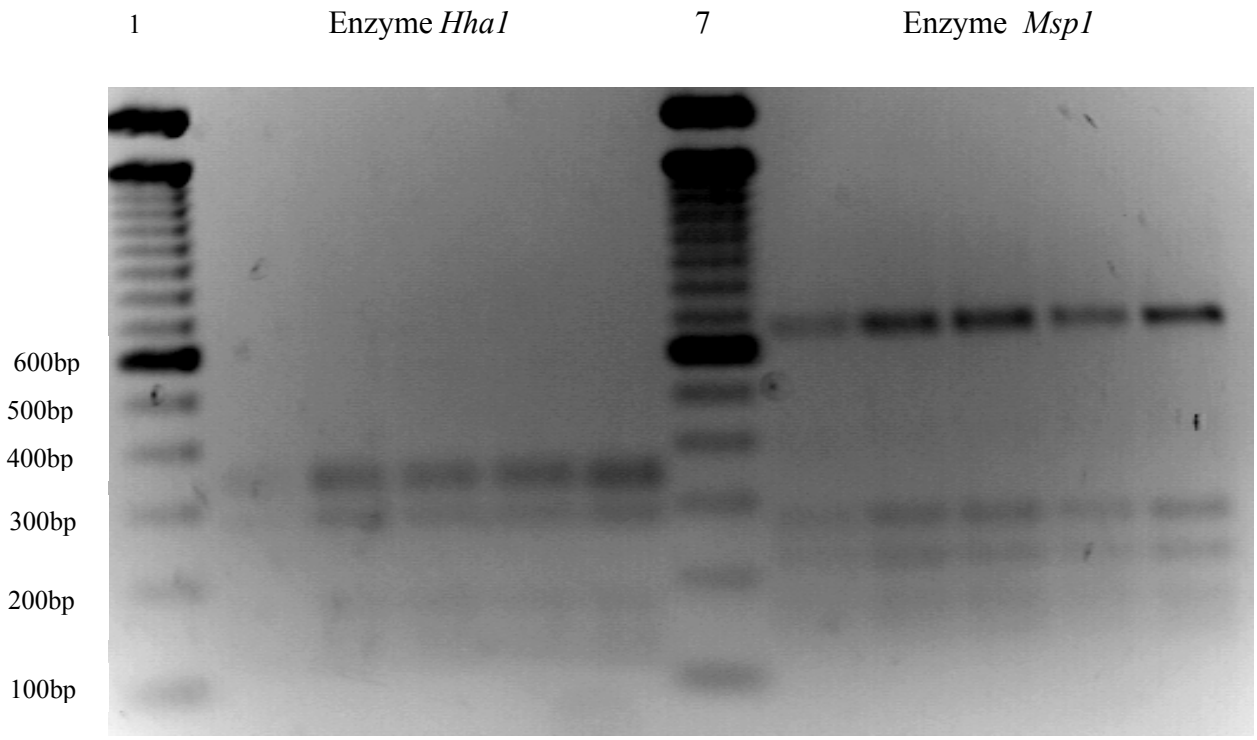


Figure 17a. 16S rDNA digested with enzyme *Hha1* and *MspI* for the standard strain *Agrobacterium tumefaciens* compared with the non symbiotic strains, lanes 1 is 100 bp ladder, from left to the right: EBRI 6, 20, 23, 25 and *A. tumefaciens*, lane 7 is 100 bp ladder, EBRI 6, 20, 23, 25 and *A. tumefaciens*.

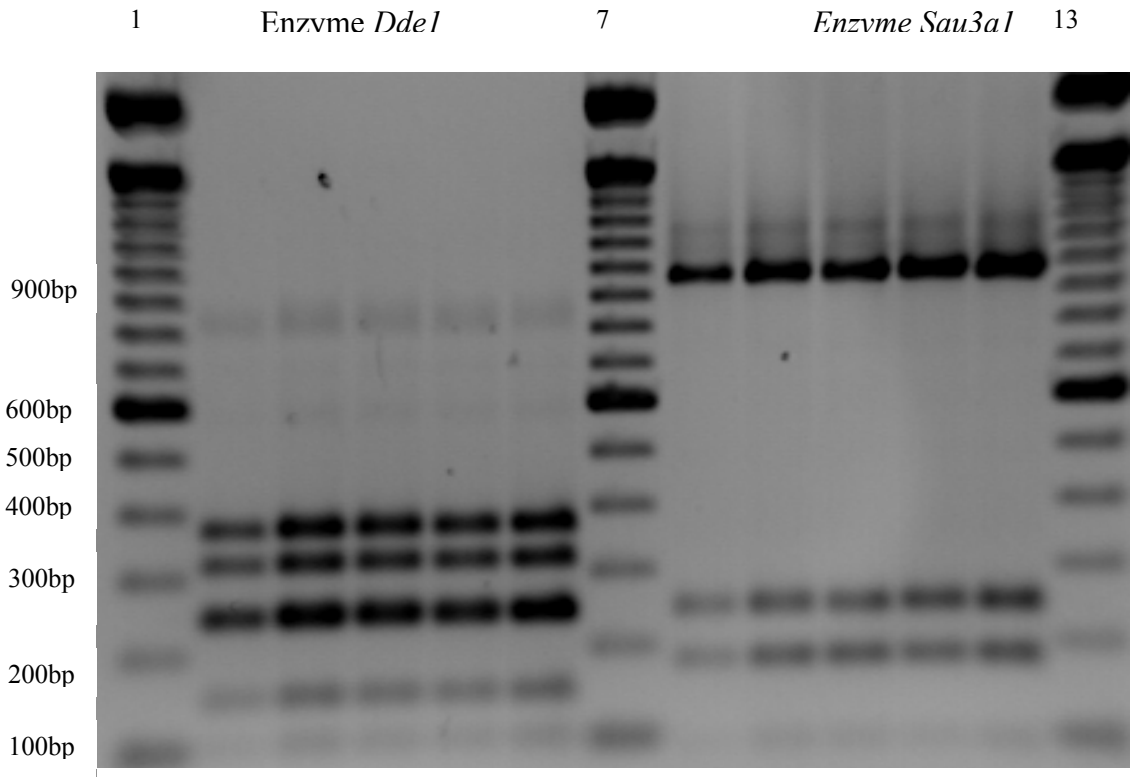


Figure 17b. 16S rDNA digested with enzyme *Dde1*, and *Sau3a1* for the standard strain *Agrobacterium tumefaciens* compared with the non symbiotic strains, lane 1 is 100 bp ladder, from left to right: EBRI 6, 20, 23, 25 and *A. tumefaciens*, lane 7 is 100 bp ladder, EBRI 6, 20, 23, 25, *A. tumefaciens* and lane 13 is 100 bp ladder.

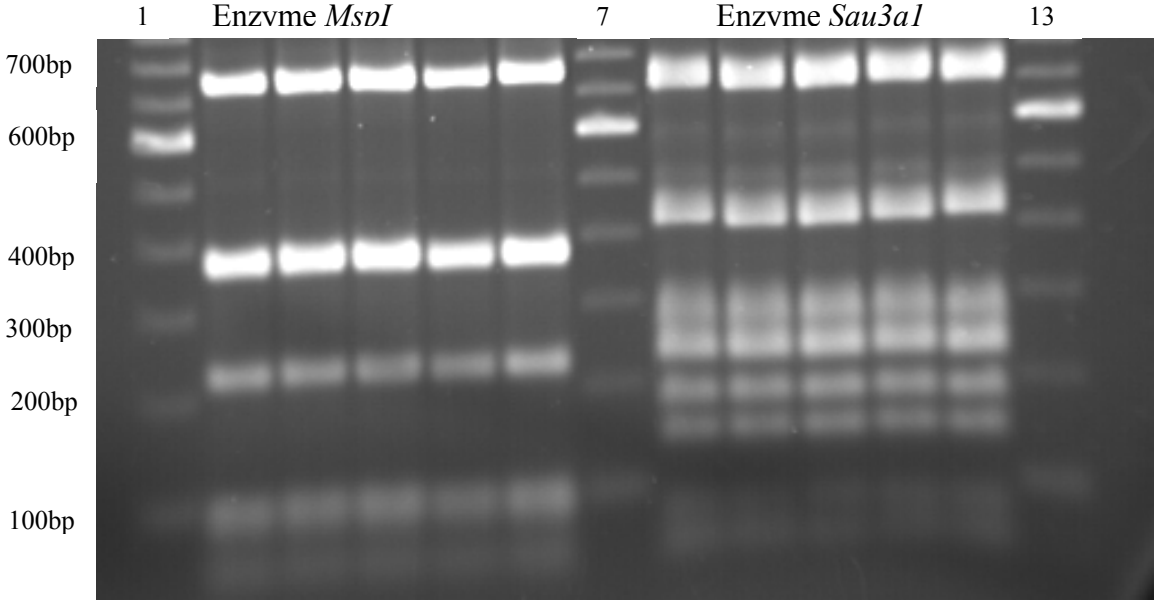


Figure 18a. 23S rDNA digested with enzyme *MspI*, and *Sau3a1* for the standard strain *Agrobacterium tumefaciens* compared with the non symbiotic strains, lanes 1 is 100 bp ladder, from left to right: EBRI 6, 20, 23, 25 and *A. tumefaciens*, lane 7 is 100 bp ladder, EBRI 6, 20, 23, 25, *A. tumefaciens* and lane 13 is 100 bp ladder.

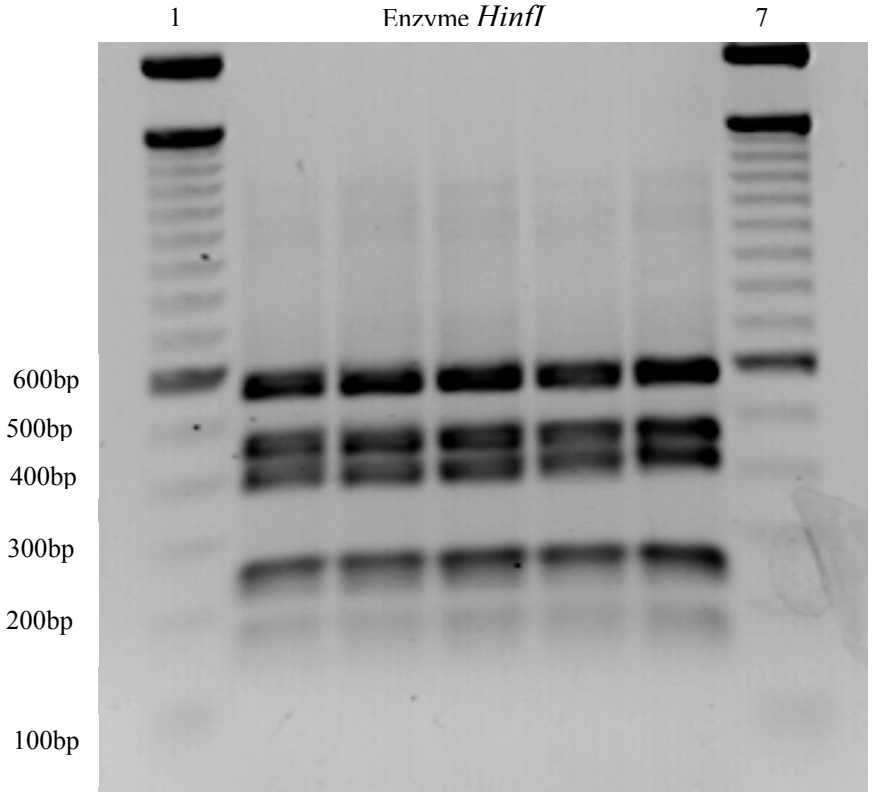


Figure 18b. 23S rDNA digested with enzyme *HinfI* for the standard strain *Agrobacterium tumefaciens* compared with the non symbiotic strains, lanes 1 is 100 bp ladder, from left to right: EBRI 6, 20, 23, 25, *A. tumefaciens* and lane 7 is 100 bp ladder.

The second experiment was the amplification of the gene coding for *nodC*. Results in Figure 19 indicate that all the strains examined had *nodC* fragment except the strains identified as *Agrobacterium tumefaciens* (EBRI 6, 20, 23 and 25). The absence of the *nodC* fragment from these strains supported their identification as non symbiotic strains. Strains EBRI 21, 27 and *R. giardinii* H152 gave only one amplified fragment, while strains EBRI 2, 3, 24, 26, 29, 32, *R. tropici* CIAT899 and *R. gallicum* R602sp produced two amplified fragments.

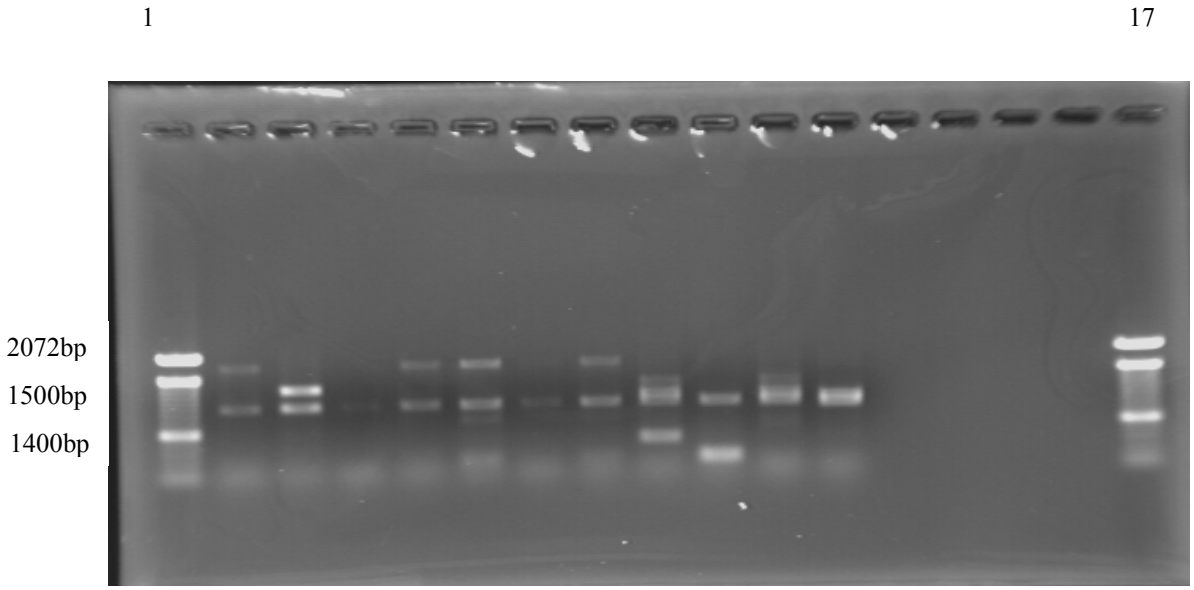


Figure 19. Amplification of *nodc* fragments, lane 1 and 17 are 100 bp ladders, from left to right:. strains EBRI 2, 3, 21, 24, 26, 27, 29, 32, *R. tropici* CIAT899, *R. gallicum* R602sp and *R. giardinii* H152 gave amplified fragments while strains EBRI 6, 20, 23 and 25 failed to amplify this gene.

3.2.6 Combination between the RFLP analysis of 16S rDNA and 23S rDNA

Results in Figure 20 summarizes the combination between the restriction patterns of rrs with four tetrameric enzymes (*HhaI* , *DdeI* , *MspI* and *Sau3aI*) and restriction patterns of rrl with three enzymes (*HinfI*, *MspI* and *Sau3aI*). The Egyptian bean rhizobial isolates could be classified into three DNA fingerprinting types. Genotype A contained strains EBRI 2, 3, 21, 24, 26, 27 and 29 which was closest to strain *R. etli* CFN42. Genotype B included strain EBRI 32 similar to *R. gallicum* R602sp. Genotype C included the *Agrobacterium*-like isolates EBRI 6, 20, 23 and 25 and the standard strain *Agrobacterium tumefaciens* 30150.

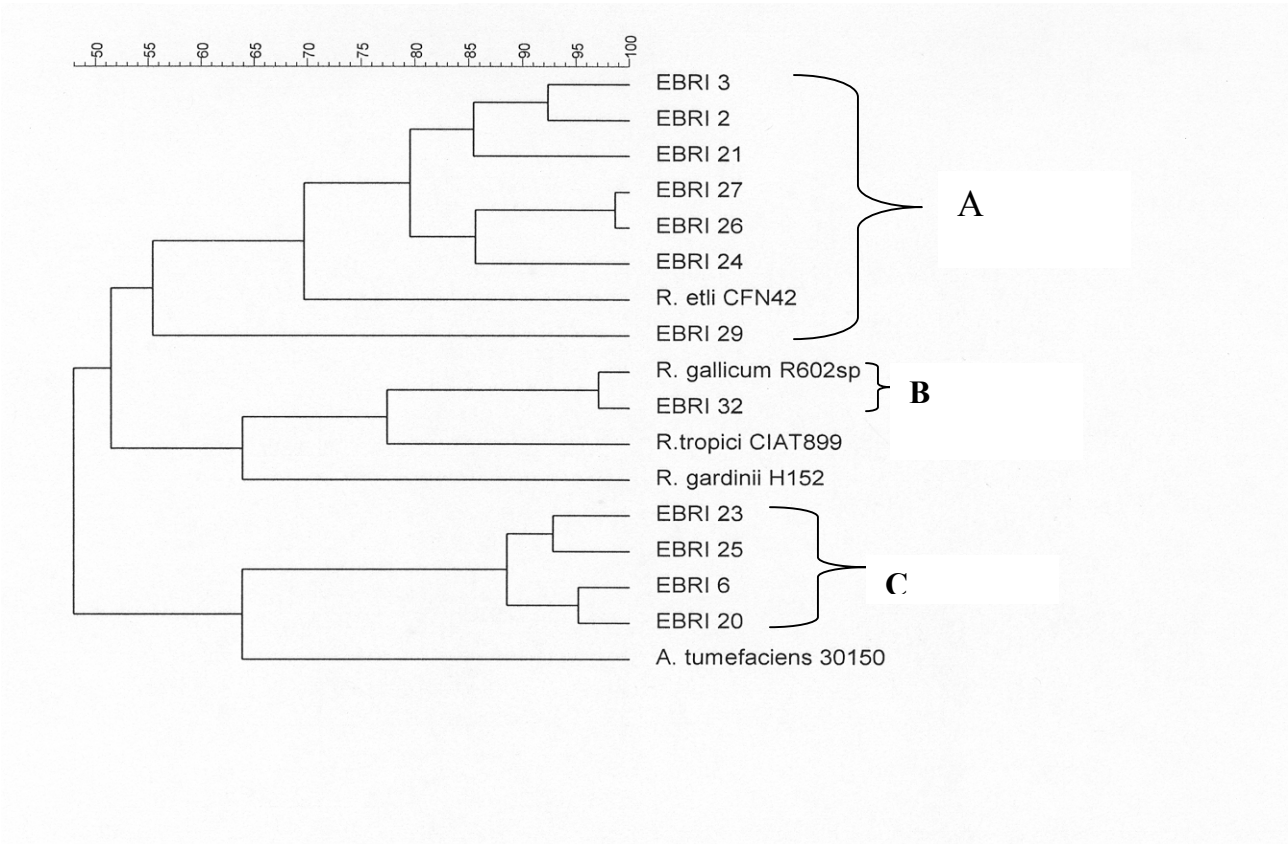


Figure 20. UPGMA dendrogram analysis of rhizobial isolates compared with standard type strains based on the combination between restriction analysis of 16S rDNA with enzymes *HhaI, DdeI, MspI and Sau3AI* and 23S rDNA with enzymes *HinfI , MspI and Sau3AI.*

3.2.7 Phylogenetic analysis of rrs sequences

Figure 21 shows the phylogenetic placement of eight Egyptian bean nodule isolates in context with a large neighbour-joining (NJ) phylogeny from the rrs sequences of all rhizobial type strains and those from closely related non-rhizobial species. Due to the bias in base composition and transitional substitution present in the data set of Tamura-Nei (TrN93), a model of nucleotide substitution was used to account for these facts, providing a highly significant (P<0.000001:DF=2) improvement in the fit of the model to the data (-LnLTrN93=7509.9307) over the Jukes-Cantor (JC) model

(LnLJC=7615.3545). Model fitting was further improved with very high statistical significance (LRT=212.0586; P<0.00001) by accounting for among-site rate variation using a discrete gamma distribution with 8 rate categories, with an estimated shape parameter value of a=0.15 (LnLTrN+G=7403.9014). Therefore the NJ phylogeny shown in Figure 21 was reconstructed using the TrN+G model. This phylogeny confirms the ARDRA results. Strains EBRI 2, 3, 21, 24, 26 and 27 form a highly supported clade along with *R. etli* CFN 42. Strains EBRI 20 and 25 are significantly associated to the lineages of *R. radiobacter* and *R. rubi* (formerly *Agrobacterium tumefaciens* and *A. rubi*). Strain EBRI 32 is closest to *R. gallicum* R602sp in ARDRA figure, and is able to nodulate *Phaseolus vulgaris* as well as *Leucaena leucocephala*, made ancestor clade with *R. etli*.

3.2.8 Hybridization with the *nifH* gene

Strains EBRI 2, 3, 21, 24, 26, 27, 29 and 32 were examined with a *nifH* probe, in order to estimate the copy number of *nifH* gene for each strain. Strains EBRI 2, 3, 21, 24, 26, 27 and 29, identified as *R. etli*, had three copies of *nifH* gene. This also supported the identification of these strains as *R. etli* bv. *phaseoli*. Strain EBRI 32 nodulating *Phaseolus vulgaris* and *Leucaena leucocephala*, had only one copy of *nifH* gene Figure 22. This confirmed the identification of this strain as *R. gallicum* bv. *gallicum*. The number of copies of *nifH* gene and their sizes are listed in Table A7.

rrs phylogeny
NJ-TrN+G
α= 0.15
1000 bootstrap replicates
52 taxa
1276 sites, complete gap deletion

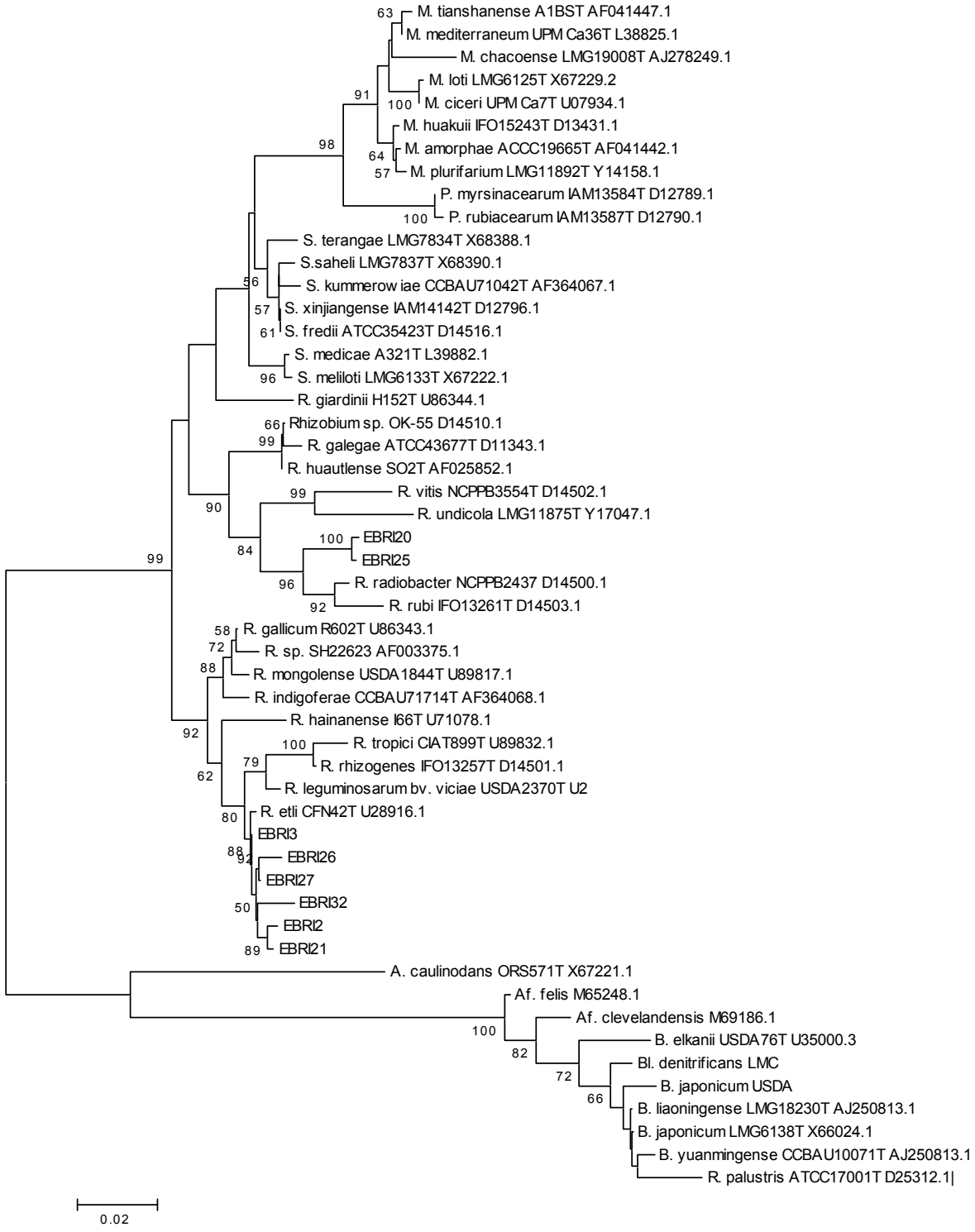


Figure 21. Phylogenetic (neighbor-joining) tree based on 1300 bp aligned sequence of 16S rDNA. Bootstrap probabilities of more than 50% are indicated at the branching points. Numbers on the left side are the accession numbers for the strains used.

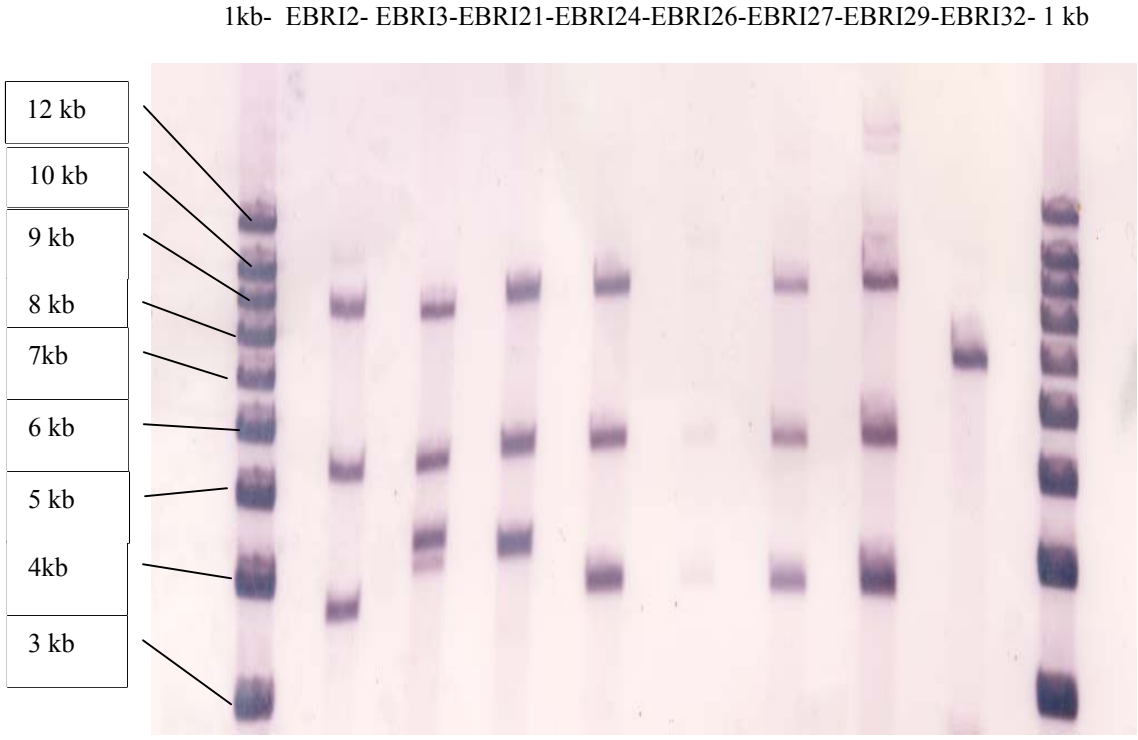


Figure 22. Southern hybridization analysis with probe *nifH* gene for genomic DNA of rhizobial strains digested with enzyme BamHI, 1 Kb from Stratagene was loded on both sides.

3.3 Phenotypic characteristics of rhizobial strains

Results in Table 9 summarized the growth of rhizobial strains on different media. Strains EBRI 2, 3, 21, 24, 26, 27 and 29, identified as *R. etli*, were able to grow on MM medium with malate as a main carbon source but were unable to grow on the same medium with sorbitol and were not also able to survive on LB medium. The *Agrobacterium*-like isolates were able to grow on LB medium and were resistant to 5 μg ml^{-1} spectinomycin. Strain EBRI 32 was unable to grow on LB medium or in basal medium amended with 5 μg ml^{-1} spectinomycin.

Table 9. Phenotypic characteristics of rhizobial strains on different media.

Strain	**Growth on LB medium**	**Growth on MM medium with**		**Growth on basal medium with**	
		Malate	**Sorbitol**	**D-Glucuronate**	**5 μg ml^{-1} spectinomycin**
R. etli CFN42T	-	+	-	+	-
EBRI 2	-	+	-	+	+
EBRI 3	-	+	-	+	-
EBRI 6	+	+	+	+	+
EBRI 20	+	+	+	+	+
EBRI 21	-	+	-	-	-
EBRI 23	+	+	+	+	+
EBRI 24	-	+	-	+	+
EBRI 25	+	+	+	+	+
EBRI 26	-	+	-	+	+
EBRI 27	-	+	-	-	-
EBRI 29	-	+	-	-	+
EBRI 32	-	+	+	-	-
R. gallicum R602spT	-	+	+	-	-
R. tropici CIAT899T	+	+	+	+	+
R. giardinii H152T	-	+	+	+	+

+: positive growth, -: no growth.

3.4 Physiological characteristics of strains to select the candidates adapted to environmental stresses

Soil degradation due to salinization or alkalisation is one of the most serious problems affecting fertility of soils especially in arid and semi-arid areas. Ten percent of the total degraded soils in the world are saline or alkaline soils (Surange et al., 1997). Consequently, we tested *R. etli* strains from Egyptian soils in addition to *R. tropici* CIAT 899 against different kinds of environmental stresses such as salinity, acidity, alkalinity and high temperature, in order to select the strains, resistant to one or several of these conditions. Results in Table 10 show, that among the eight strains we found a high degree of diversity, two of these strains were highly tolerant to salt up to 4% NaCl (EBRI 21 and EBRI 26). Three strains were moderately tolerant to salinity up to 3% NaCl (EBRI 24, 27 and 29). Strains EBRI 2, 3 and 32 were very sensitive to salt concentration. Their growth was suppressed by the addition of sodium chloride to the medium at 0.6% or 1% NaCl. Strain *R. tropici* CIAT 899 was able to resist levels of NaCl up to 2.5%. This strain did growth even at 4% NaCl, when the incubation time was prolonged to six days (data not shown).

The strains studied are well adapted to different pHs. At low pH, Strains EBRI 2, 21, 26, 27 and 29 were better adapted than strains EBRI 3, 24 and 32. At high pH, strains EBRI 2, 3, 27 and 32 were less tolerant than strains EBRI 21, 24, 26 and 29. A significant positive correlation was found between salt tolerance and the adaptation of rhizobial strains to alkaline conditions. This is shown in Table 10. All salt tolerant strains (2% to 4%NaCl), except strain EBRI 27, were also highly resistant to alkaline conditions.

Nodule formation in *Phaseolus vulgaris* is hampered by root temperatures above than 30 $^{\circ}$C. To identify strains adapted to high temperatures, strains were grown in liquid culture at three temperatures. All strains grew well at the optimum temperature of 30 $^{\circ}$C. Strains EBRI 2 and EBRI 26 survived well at

42 °C. Strains EBRI 3, 24, 27, 29 and 32 were able to survive at 37 °C while strain EBRI 21 did not survive.

Table 10. Phenotypic characteristics of rhizobial strains nodulating *Phaseolus vulgaris* grown under environmental stresses.

Strain	% NaCl inhibiting growth	low pH values tolerated	high pH values tolerated	maximal temperatures tolerated °C
CIAT899	3	4	9	37 °C
EBRI2	0.6	4.7	8.5	42 °C
EBRI3	0.6	5.5	8.5	37 °C
EBRI21	4.5	4.7	9	30 °C
EBRI24	3.5	5.5	9	37 °C
EBRI26	4.5	4.7	9	42 °C
EBRI27	3.5	4.7	8.5	37 °C
EBRI29	3.5	4.7	9	37 °C
EBRI32	1.0	5.5	8.5	37 °C

Strains EBRI 21 and EBRI 26 as most salt tolerant, and strain EBRI 2 as most salt sensitive were selected to study them under the combined stress of salinity and alkalinity in comparison with strain *R. tropici* CIAT 899. The effect on the viability of *Rhizobium* strain EBRI 2 was evaluated after 3 days. Strain EBRI 2 was able to survive at different levels of pH from 7.5 to 9 (Figure 23 a) compared to pH 7 under no stress of salt. Under saline conditions (2.5% NaCl, Figure 23 b) there is no growth observed for this strain at any pH.

The type strain of *Rhizobium tropici* CIAT 899 was less tolerant to the combination of salt and alkaline stress than the Egyptian strains. It was able to survive only at 2.5% NaCl and pH7 after 3 days but not at the same level of salinity with pH above than 7 (Figure 24a and b).

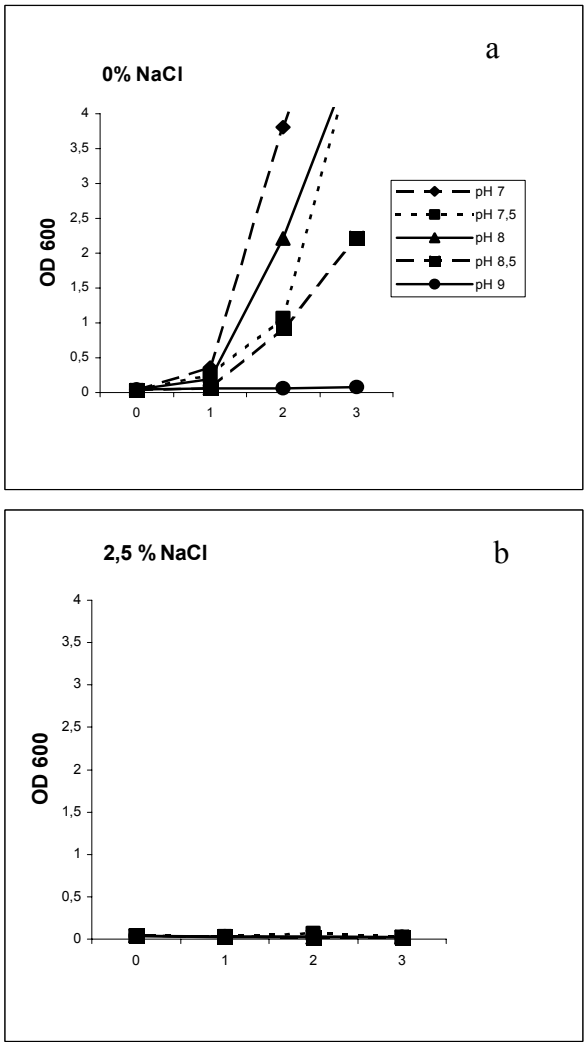


Figure 23. Growth of *Rhizobium etli* EBRI 2 at different pHs and salinity levels.

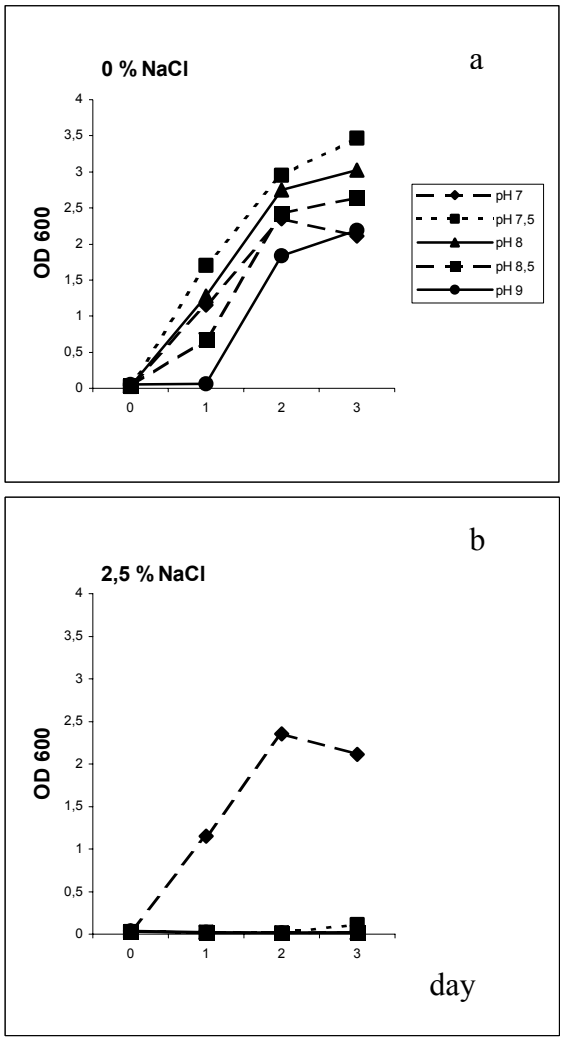


Figure 24. Growth of *Rhizobium tropici* CIAT 899 at different pHs and salinity levels.

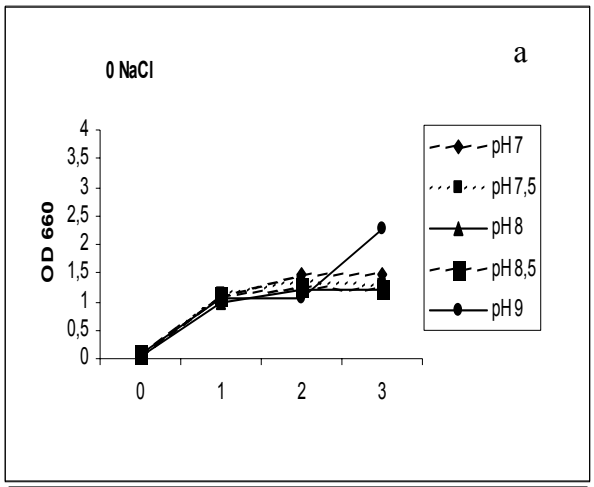


Figure 25. Growth of *Rhizobium etli* EBRI 21 at different pHs and salinity level.

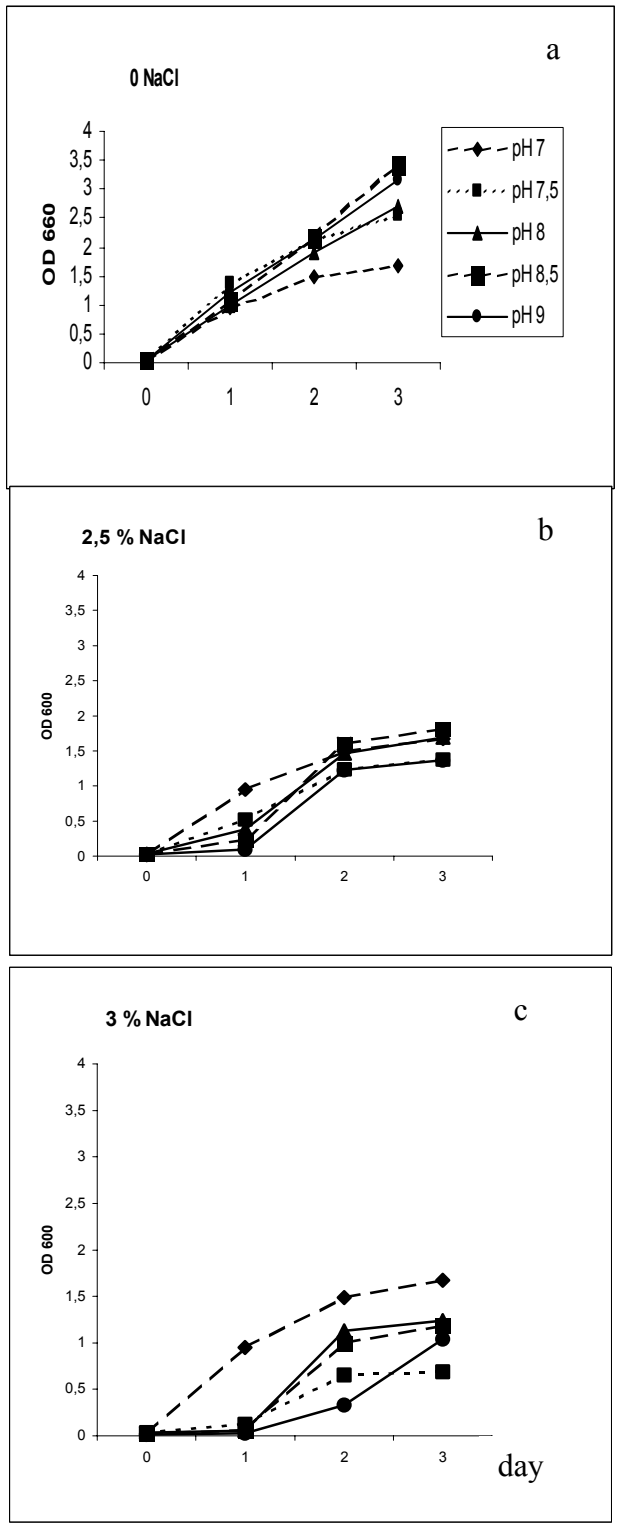


Figure 26. Growth of *Rhizobium etli* EBRI 26 at different pHs and salinity levels.

The salt resistant strain EBRI 21 survived well with 2.5 and 3% NaCl at different pH levels as compared with the growth without addition of sodium chloride (Figure 25a-c). Strain EBRI 26 has the same physiological response as EBRI 21 under stress of salt and alkaline conditions (Figure 26a-c). Both strains EBRI 21 and 26 were not able to grow at 4% NaCl at different pHs, although they were able to survive at 4% NaCl (Table 10).

The symbiotic performance of strains EBRI 2 and EBRI 26 as salt sensitive and salt resistant, was determined under salt and alkaline conditions with two cultivars of common bean. Results in Table 11 summarize the nodulation parameters of strains EBRI 2 and EBRI 26 under stress of salinity with cultivar Giza 6 and Saxa. In cultivar Giza 6, no significant difference was observed between the nodule numbers formed by salt sensitive strain EBRI 2 without stress of salt and under stress of salt (0.2% NaCl). With the increase to 0.4% NaCl, the process of nodule formation in *Phaseolus vulagris* was extremely sensitive to NaCl. A reduction in nodule number, nodule fresh weight and acetylene reduction assay by 54%, 49%, 47% was observed with the salt sensitive strain EBRI 2 at 0.4% NaCl, while with the salt resistant strain EBRI 26, the addition of sodium chloride to the nutrient solution promoted the formation of nodules at both of two levels of salinity. With cultivar Saxa, the same trend of results was observed with the two examined strains, but the nitrogenase activity was strongly depressed at 0.4% NaCl with the salt sensitive EBRI 2 and salt resistant EBRI 26 as well as (3.9 and 3.8 n mol C_2H_4 h^{-1} mg^{-1} nodules). These observations indicate that there is a correlation between strain behaviour under stress in liquid culture and the performance of these strains under symbiotic conditions. The evaluation of strains in pure culture amended with salt or at alkaline stress is a suitable method to select the highly adapted strains.

Table 11. Nodulation parameters of selected isolates of bean rhizobia with two host cultivars under stress of salinity, 21 days after planting.

Treatments	No. of nodules plant^{-1}	F W of nodules mg plant^{-1}	FW of shoots g plant^{-1}	FW of roots g plant^{-1}	n mol C_2H_4 h^{-1} mg^{-1} nodules FW
Plant cultivar Giza 6					
Control	0.0c	0.0d	3.4c	0.55	0.0f
Inoculated with EBRI 2	103ab	309ab	4.3b	0.36	29.9a
Inoculated with EBRI 2+0.2 % NaCl	123a	374a	5.4a	0.45	24.6b
Inoculated with EBRI 2+0.4 % NaCl	56ba	150cb	4.3b	0.42	13.9dc
Inoculated with EBRI 26	54ba	163cb	4.6b	0.38	26.8a
Inoculated with EBRI 26+0.2 % NaCl	108ab	335ab	5.6a	0.44	24.1b
Inoculated with EBRI 26+0.4 % NaCl	83ba	224ba	4.7b	0.44	18.2c
Plant cultivar Saxa					
Control	0.00c	0.00d	1.9d	0.50	0.0f
Inoculated with EBRI 2	81ba	239ba	3.0c	0.29	18.2c
Inoculated with EBRI 2+0.2 % NaCl	88ab	344ab	3.1c	0.47	15.7cd
Inoculated with EBRI 2+0.4 % NaCl	61ba	160cb	2.2d	0.38	3.8e
Inoculated with EBRI 26	66ba	267ba	3.3c	0.25	19.8c
Inoculated with EBRI 26+0.2 % NaCl	113ab	357ab	2.9c	0.34	17.4c
Inoculated with EBRI 26+0.4 % NaCl	89ab	224ba	2.6c	0.32	3.9e
L.S.D. 0.05	**37.31**	**96.74**	**0.59**	**N.S.**	**3.56**

FW: fresh weight.

Results in Table 12 summarize the symbiotic efficiency of strains EBRI 2 and EBRI 26 under alkaline conditions. Strains EBRI 2 and EBRI 26 were able to nodulate and fix nitrogen with cultivars Giza 6 and Saxa at pH 8 and pH 8.5. Alkalinity has less detrimental effect on nodule formation and nitrogen fixation compared with salinity Table 11. It is surprising that the activity of acetylene

reduction increased with both strains and both cultivars under high alkaline conditions compared with the neutral pH.

Table 12. Nodulation and Nitrogen fixation under stress of alkalinity with two host cultivars, 21 days after planting.

Treatments	**No. of nodules plant^{-1}**	**F W of nodules mg plant^{-1}**	**F W of shoots g plant^{-1}**	**FW of roots g plant^{-1}**	**n mol C_2H_4 h^{-1} mg^{-1} nodules FW**
Plant Cultivar Giza 6					
Control	0.0c	0.0d	3.00b	0.14c	0.0c
Inoculated with EBRI 2	70a	147cb	4.4a	0.16bc	14.9b
Inoculated with EBRI 2 + pH 8	67ba	189b	4.1a	0.19ba	24.2a
Inoculated with EBRI 2 + pH 8.5	50ba	127cb	3.6ba	0.23a	19.3ba
Inoculated with EBRI 26	69a	188b	4.4a	0.23a	23.7a
Inoculated with EBRI 26 + pH 8	67ba	206b	4.8a	0.25a	27.2a
Inoculated with EBRI 26 + pH 8.5	52ba	181b	3.7ba	0.21a	24.9a
Plant cultivar Saxa					
Control	0.0c	0.0d	2.1c	0.14c	0.0c
Inoculated with EBRI 2	70a	177b	2.4bc	0.12c	12.8b
Inoculated with EBRI 2 + pH 8	91a	253a	2.8b	0.14c	11.6b
Inoculated with EBRI 2 + pH 8.5	63ba	170b	2.8bc	0.13c	20.4ba
Inoculated with EBRI 26	79a	292a	2.8bc	0.12c	16.2b
Inoculated with EBRI 26 + pH 8	72a	200b	2.5bc	0.11c	18.8ba
Inoculated with EBRI 26 + pH 8.5	59ba	152b	2.7bc	0.15bc	25.7a
L.S.D. 0.05	**21.35**	**42.72**	**0.69**	**0.04**	**5.79**

FW: fresh weight.

3.5 Selection of competitive strains of *Rhizobium,* nodulating *Phaseolus vulgaris* and adapted to soil stress conditions in Egypt, using *Gus*-reporter gene technique

To select the most competitive strains, we used the *Gus* reporter gene technique. The first step was the construction of a *Gus* reporter gene strain. Strain *R. tropici* CIAT 899G with the *Gus* gene produced blue colonies compared with the wild type when grown on MM amended with 50 µg ml^{-1} x-gluc (Figure 27).

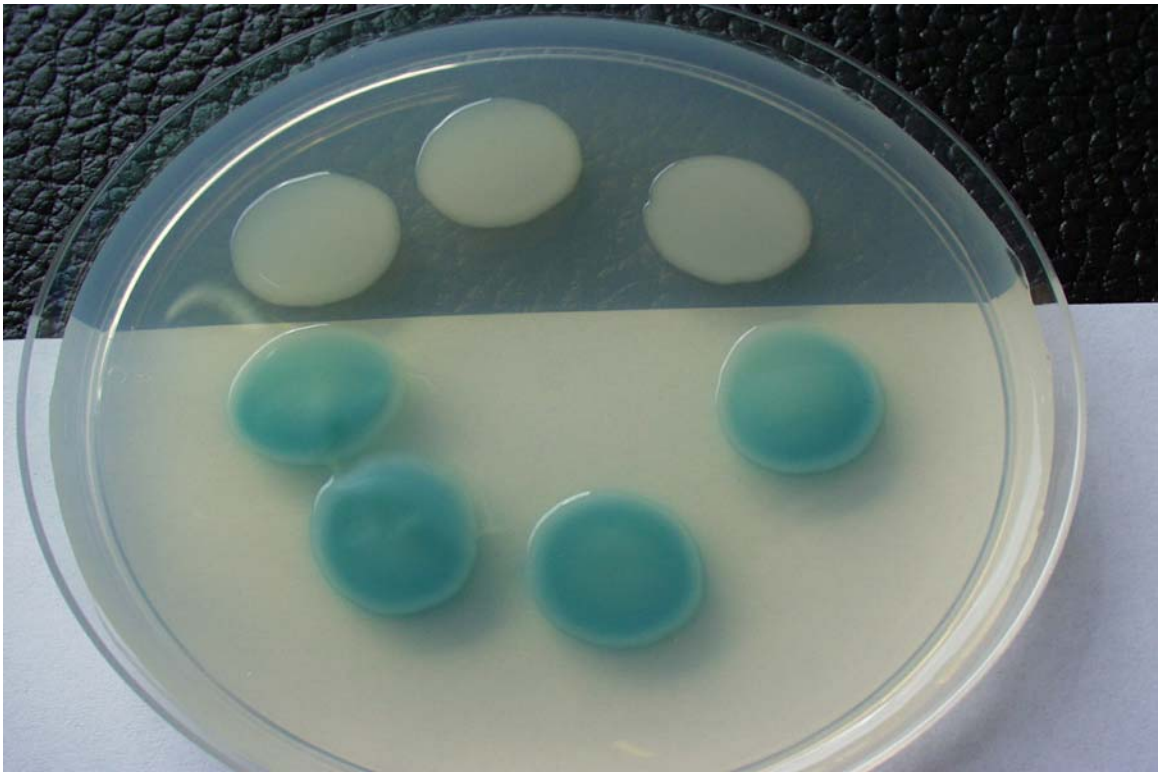


Figure 27. Blue colonies of strain *R. tropici* CIAT 899G compared with white colonies from wild type grown on MM amended with x-gluc substrate.

No statistically significant differences were detected in nodule number among plants inoculated with CIAT 899G or the parent strain CIAT 899 (Table 13).

Table 13. Nodulation and nitrogen fixation of single or dual strain inoculum with two host cultivars, 30 days after inoculation (pH 6.85).

Treatments	No. of nodules plant^{-1}	FW nodules mg plant^{-1}	FW of shoots g plant^{-1}	FW of roots mg plant^{-1}	n mol C_2H_4 h^{-1} mg $^{-1}$ nodules FW (ARA activity)
Bean cultivar Giza 6					
Control	0.0b	0.0d	4.6d	440	0.0c
Inoculated with CIAT 899Wt	229a	1103b	7.9b	390	13.7ab
Inoculated with CIAT 899G	236a	1648a	13.2a	520	6.9b
Inoculated with EBRI 2	231a	1055b	10.3b	450	13.5ab
Inoculated with EBRI 26	214a	963b	10.2b	450	11.6b
Inoculated with CIAT 899G + EBRI 2	200a	675cb	7.5cb	470	16.2ab
Inoculated with CIAT 899G +EBRI 26	139a	610cb	8.1bc	550	15.3ab
Bean Cultivar Saxa					
Control	0.0b	0.0d	2.7ed	450	0.0c
Inoculated with CIAT 899 Wt	178a	795bc	4.9d	380	11.3b
Inoculated with CIAT 899G	147a	563cb	5.4cd	420	14.8ab
Inoculated with EBRI 2	159a	616cb	5.1d	360	15.5ab
Inoculated with EBRI 26	169a	605cb	5.5cd	410	17.5a
Inoculated with CIAT 899G + EBRI 2	221a	748bc	6.1cd	480	11.7ab
Inoculated with CIAT899G + EBRI 26	173a	512cb	5.1d	460	15.9a
L.S.D. 0.05	**N.S**	**391.69**	**2.27**	**N.S**	**5.18**

Wt wild type, CIAT899G marked with *gene*-gene, EBRI Egyptian bean rhizobial isolates, N. S. non significant, Letters on the right side indicating the statistical analysis, FW: fresh weight.

The number of nodules developed by inoculation with Egyptian strains EBRI 2 and EBRI 26 were not affected by co-inoculation with strain CIAT 899G (Table 13). This result was obtained with the cultivar Giza 6 from Egypt as well as with the cultivar Saxa from Europe. It is remarkable, that the root weight of both

cultivars is almost the same in the non inoculated control plants as in those plants, which produce after symbiotic infection a large amount of nodules. The weight of the nodules exceeds the weight of the roots by a factor of two or more. However, this ratio was significantly lower, when a co-inoculation of the strain EBRI 2 or EBRI 26 with the CIAT 899G was used. This effect was only observed with the Egyptian cultivar Giza 6. The specific ARA activity per plant was rather similar in all variations. The higher nodule weight in the plants inoculated with CIAT 899G produced a lower specific activity per mg nodule weight.

Figure 28. Nodule staining with x-gluc substrate to detect blue nodules from CIAT 899G marked with Gus gene and white nodules from Egyptian bean rhizobial isolates

To estimate the nodule occupancy, roots with dual strain inoculation were incubated in *gus* staining buffer to recognize the blue and white nodules. The staining of nodules was strong (Figure 28) and it was easy to count the blue and white nodules, in order to estimate the percentages of nodule occupancy. Results

of competition between the Egyptian strains *R. etli* EBRI 2 or EBRI 26 with the strain CIAT 899G under neutral pH are presented in Table 14.

Table 14. Nodule occupancy of *R. etli* EBRI 2 and EBRI 26 at neutral pH, 30 days after inoculation in competition with *R. tropici* CIAT 899G marked with *gus* gene.

Strains used	**% Nodule occupancy by EBRI 2**	**% Nodule occupancy by CIAT 899G**	**% Dual nodule occupancy**	**Competitiveness index C X:Y**	**Host cultivar**
CIAT 899G+EBRI2	52.1±3.5	46.8 ±4.5	1.1±0.6	0.05	Saxa
CIAT 899G+EBRI2	66±3.2	33.39 ±1.8	0.61 ±0.6	0.29	Giza 6
	% Nodule occupancy by EBRI 26	**% Nodule occupancy by CIAT 899G**	**% Dual nodule occupancy**	**Competitiveness index C X:Y**	**Host cultivar**
CIAT 899G+EBRI26	61.1±3.57	38.9±2.6	0.00	0.19	Saxa
CIAT 899G+EBRI26	67.5 ±2.9	31.3±2.8	1.2 ±0.53	0.33	Giza 6

X: EBRI strain, Y: CIAT 899G, Results are means of three replicates ± standard errors.

Strain EBRI 2 is a strong competitor against strain CIAT 899G only in cultivar Giza 6 (66% nodule occupancy), strain EBRI 26 was also very competitive with 67.5% nodule occupancy. A small percent of nodules (0.61-1.2%) were partially staining blue, indicating a double nodule occupancy. Competitiveness indices were estimated using the equation described by Beattie et al. (1989). The results indicate that the Egyptian rhizobial strains are highly competitive against CIAT 899G.

Results in Table 15 present the nodulation parameters of single or dual strain inoculation under stress of salinity using the cultivar Giza 6. There was no significant differences among nodule numbers formed. The amount of nodule fresh weight, never exceeded the root fresh weight by more than a factor of 2. The ARA activity per plant was again rather similar in all combinations. Generally, the stress of salinity decreased the nodule number, nodule fresh

weight and shoot fresh weight compared with the growth of plants under normal conditions (Table 13).

Table 15. Nodulation and nitrogen fixation of bean cultivar Giza 6 under stress of salinity (34.2 mM NaCl), 25 days after inoculation.

Treatments	**No. of nodules plant^{-1}**	**FW of nodules mg plant^{-1}**	**FW of shoots g plant^{-1}**	**FW weight of roots mg plant^{-1}**	**n mol C_2H_4 h^{-1} mg $^{-1}$ nodules FW (ARA activity)**
Control	0.0b	0.0c	3.4c	350	0.0c
Inoculated with CIAT899Wt	69a	390ba	5.6ba	470	12.4a
Inoculated with CIAT899G	97a	641a	7.6a	430	10.3a
Inoculated with EBRI2	144a	706a	7.7a	430	8.9ba
Inoculated with EBRI26	98a	568a	6.9a	420	10.2a
Inoculated with EBRI2+899G	82a	460a	6.8a	420	10.8a
Inoculated with EBRI26+899G	83a	343ba	7.4a	360	13.3a
L.S.D. 0.05	**N.S**	**284.69**	**1.52**	**N.S**	**3.55**

Wt wild type, CIAT899G marked with *gus* gene, N. S. non significant, Letters on the right side indicating on the statistical analysis, FW: fresh weight.

The percentages of nodule occupancy under stress of salinity are listed in Table 16. It is interesting to note that the salt sensitive strain EBRI 2 (tolerance up to 0.5% NaCl) was a better competitor against CIAT 899G than the highly resistant strain EBRI 26 (tolerant up to 4% NaCl). Strain EBRI 2 occupied 87.4% of the nodules while strain EBRI 26 occupied only 63.7% against CIAT 899G.

Table 16. Nodule occupancy of *R. etli* EBRI 2 and EBRI 26 under stress of salinity or alkalinity with bean cultivar Giza 6, 25 or 30 days after inoculation competing with *R. tropici* CIAT 899G marked with *gus* gene.

Strains used	**% Nodule occupancy by EBRI 2 or EBRI 26**	**% Nodule occupancy by CIAT 899G**	**% Dual nodule occupancy**	**Competitiveness index C X:Y**	**Stress of salinity**
CIAT 899G+EBRI2	87.4±2.5	9.4±1.8	3.2±0.76	0.86	34.2 mM NaCl
CIAT 899G+EBRI26	63.7±5.2	33.1±4.3	3.2±1.3	0.27	34.2 mM NaCl
	Nodule occupancy by EBRI 2 or EBRI 26	**Nodule occupancy by CIAT 899G**	**Dual nodule occupancy**	**Competitiveness index C X:Y**	**Stress of alkalinity**
CIAT 899G+EBRI2	83±4.4	12.4±2.5	4.6±1.7	0.71	pH 8
CIAT 899G+EBRI26	53.2±5.8	43.9±5.8	2.9±1.4	0.08	pH 8

X: EBRI strain, Y: CIAT 899G, Results are means of three replicates ± standard errors.

Results in Table 17 give nodulation parameters of single or mix strains inoculation under stress of alkalinity. In all cases the nodule numbers was higher than under salt stress (Table 15). The percentages of nodule occupancy are listed in Table 16. Although the strains EBRI 26 and CIAT 899G are more resistant to alkalinity in liquid culture (maximum level pH 9) than strain EBRI 2 which was moderately tolerant to alkaline pH (maximum level of growth pH 8.5), strain EBRI 2 gave the highest percent of nodule occupancy 83% compared with EBRI 26 (53.2%) against CIAT 899G (12.4% and 43.9%) respectively.

Table 17. Nodulation and nitrogen fixation of bean cultivar Giza 6 under stress of alkalinity at pH 8, 30 days after inoculation.

Treatments	No. of nodules plant^{-1}	FW of nodules mg plant^{-1}	FW weight of shoots g plant^{-1}	FW of roots mg plant^{-1}	n mol C_2H_4 h^{-1} mg^{-1} nodules FW (ARA activity)
Control	0.0c	0.0c	4.6b	510a	0.0c
Inoculated with CIAT899Wt	158ab	1156a	9.7a	560a	14.2b
Inoculated with CIAT899G	163ab	980a	9.5a	480ba	14.9b
Inoculated with EBRI2	136ba	671ab	8.4ab	520a	19.8a
Inoculated with EBRI26	127ba	657b	7.9ab	480ba	19.2a
Inoculated with EBRI2+899G	159ab	930a	8.4ab	520a	14.3b
Inoculated with EBRI26+899G	182a	675ba	6.5ba	430b	16.6ab
L.S.D. 0.05	**40.1**	**269.81**	**2.01**	**0.08**	**3.39**

Wt wild type, CIAT899G marked with *gus* gene, Letters on the right side indicating on the statistical analysis, FW: fresh weight.

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Soil temperature is a major factor determining rhizobial survive, the temperature can exceed that 40 °C in the summer season, affecting the growth, survival and symbiotic function of rhizobial strains. Therefore, it is important to select competitive strains at high temperatures. In order to obtain the highly competitive strains under this condition, strains EBRI 2 and EBRI 26 were examined against strain of CIAT 899G at 35 °C. Results in Table 18 present the data of nodulation by these strains under elevated temperature. Generally, increasing temperature affected nodulation more than salinity stress. This was evident from the reduction of nodule number and nodule fresh weight, and severely acetylene reduction. No significant differences was observed for the shoot weights, indicating that under these conditions, the inoculated plants suffered from the deficiency of available nitrogen like the un-inoculated plants. Inoculated and non-inoculated plants were chlorotic.

Table 18. Competition under stress of temperature 35 °C, 25 days after inoculation (Cultivar Giza 6).

Treatments	No. of nodules plant^{-1}	FW of nodules mg plant^{-1}	FW of shoots g plant^{-1}	FW of roots g plant^{-1}	n mol C_2H_4 h^{-1} mg^{-1} nodules FW
Control	0.0	0.0	4.4	0.62	0.00
Inoculated with CIAT899Wt	28	113	3.6	0.66	3.1
Inoculated with CIAT899G	29	131	3.9	0.65	3.6
Inoculated with EBRI2	24	68	4.3	0.68	3.2
Inoculated with EBRI26	34	106	3.8	0.63	3.2
Inoculated with EBRI2+899G	21	86	4.6	0.61	3.1
Inoculated with EBRI26+899G	45	285	4.0	0.66	3.3
L.S.D. 0.05	**N.S**	**152.4**	**N.S**	**N.S**	**N.S**

Wt wild type, CIAT899G marked with *gus* gene, N. S. none significant, FW: fresh weight.

As obtained before, strain EBRI 2 was the best competitor in nodule occupancy against CIAT 899G Table 19. Strain EBRI 2 had 81.5% while strain EBRI 26 occupied 64.7% compared with strain CIAT 899G with 18.5% and 33% of nodule occupancy respectively. The nodules seemed to be ineffective nodules, observed from general appearance of the plants and also by checking the leghemoglobin inside the nodules.

Table 19. Nodule occupancy of *R. etli* EBRI 2 and EBRI 26 under stress of temperature 35 °C with bean cultivar Giza 6, 25 days after inoculation competing with *R. tropici* CIAT 899G marked with *gus* gene.

Strains used	% Nodule occupancy by EBRI 2 or EBRI 26	% Nodule occupancy by CIAT 899G	% Dual nodule occupancy	Competitiveness index C X:Y	Stress of temperature
CIAT 899G+EBRI2	81.5%±3.3	18.5%±3.3	0.0	0.64	35 °C
CIAT 899G+EBRI26	64.7%±5.1	33%±7.8	2.3±1.6	0.27	35 °C

3.6 Effect of nitrogen fertilizer application on nodulation of *Phaseolus vulgaris*

Under the intensification of agriculture especially in developing countries to meet the excess demands for food, farmers use more nitrogen fertilizer in their farming systems which prevent or limit the response of rhizobial inoculation under field conditions. Therefore we aimed in this experiment to know at which level of nitrogen fertilization, still the rhizobial strains have the ability to nodulate common bean cultivars efficiently.

Results in Table 20 show, that the nodulation was markedly reduced with the increase of nitrogen fertilizer dose. This negative effect was observed with both strains examined in this experiment. The nodule records were 93 and 120 nodule plant^{-1} with strains EBRI 2 and EBRI 26 without nitrogen application, while these numbers decreased significantly to 52 and 38 nodules plant^{-1} at 80 ppm N rate.

The same results were obtained for nodule biomass with 512 mg without nitrogen addition compared to 173 mg plant^{-1} at 80 ppm N. At the application of 160 ppm N, there are no nodules observed indicating that the nodulation was suppressed completely.

Rhizobial inoculation and nitrogen fertilization supported higher shoot biomass production compared with the un-inoculated and un-fertilized plants. The application of nitrogen fertilizer dose of 160 ppm N was the most efficient in this respect. The effectiveness values gave indication that the inoculation with EBRI 2 plus 20 or 40 ppm N gave the best value (68.1%) compared with the full N dose (100%) with a good values also for nodule number, nodule and shoot weight and acetylene reduction assay. Generally, the parameter of acetylene reduction decreased gradually depending on the dose of nitrogen fertilizer used.

Strikingly, a low dose of nitrogen (20 ppm) promoted the nitrogen fixation (20.4 n mol C_2H_4 h^{-1} mg nodules) compared with the inoculation without nitrogen fertilization (17.9 n mol C_2H_4 h^{-1} mg nodules) by both strains.

Ultimately, we can conclude that the application of 20 or 40 ppm N in addition to rhizobial inoculation has not much negative effects on nodulation and nitrogen fixation for this crop.

Table 20. Nodulation and nitrogen fixation parameters of cultivar Giza 6 with the application of nitrogen fertilizer, 25 days after inoculation.

Treatments	**No. of nodules plant^{-1}**	**FW of nodules mg plant^{-1}**	**FW of shoots g plant^{-1}**	**Effectiveness %**	**n mol C_2H_4 h^{-1} mg^{-1} nodules FW**
Control	0.0e	0.0d	5.1cb	-	0.0c
Full dose N (160 ppm) + no inoculation	0.0e	0.0d	14.4a	**100**	0.0c
Inoculated by EBRI2	93b	512a	8.3b	57.96	17.9a
Inoculated by EBRI2+20 ppm N	69cb	375b	9.8b	68.2	**20.4a**
Inoculated by EBRI2+40 ppm N	71cb	355b	9.8b	68.1	12.9ba
Inoculated by EBRI2+80 ppm N	52d	173c	14.9a	**103.4**	10.7b
Inoculated by EBRI2+160 ppm N	0.0e	0.0d	15.7a	**109**	0.0c
Inoculated by EBRI 26	120a	488a	6.8cb	47.2	17.7a
Inoculated by EBRI26+20 ppm N	77b	286b	7.5b	52.1	**21.0a**
Inoculated by EBRI26+40 ppm N	82b	254cb	8.6b	59.7	12.2b
Inoculated by EBRI26+80 ppm N	38d	125c	10.0b	69.4	8.6b
Inoculated by EBRI26+160ppm N	0.0e	0.0d	16.9a	**117.4**	0.0c
L.S.D 0.05	16.4	96.4	2.75	-	5.29

Effectiveness %= fresh weight of inoculated plants or inoculated plus fertilized plants ÷fresh weight of full fertilized plants, FW: fresh weight.

3.7 Proteom analysis of salt tolerance in *Rhizobium etli* strain EBRI 26

The salt tolerant strain *R. etli* EBRI 26, was selected to study the changes in proteom profiles after salt stress. The 2-D gel electrophoresis was used to analyse proteins expressed by this strain under stress of salt compared to normal condition. Total soluble protein was isolated from *R. etli* strain EBRI 26 grown in 20E liquid medium without addition of salt or with the addition of 4% NaCl. The concentration of protein was 6.5 mg ml^{-1} and 6.1 mg ml^{-1} for strain grown under control and stress conditions. Spots were detected by two methods. In the first method, spots of protein were recognized by staining in CBB dye, in the second method spots were detected by scanning the gel with a Typhoon scanner after labelling them with CyDyes. For CBB staining, in the first dimension 700 µg of protein from each sample were separated by isoelectric focusing in immobilized pH gradient IPG (pH 4-7). In the second dimension, the samples were electrophorsed on 12% PAGE-SDS. Six spots of proteins were easily recognized as over-expressed, after the application of 4% NaCl stress (Figure 29). These proteins have Ip (Isoelectric points) between 5 to 5.5 and masses of approximately 22, 25, 40, 65, 70 and 95 KDa respectively. The spots were picked up and digested with trypsin and analysed by MALDI-TOF analysis. The MFP (mass finger print) of these proteins are documented in Figure A2. Although, we obtained a good MFP, we could not identify these proteins, because the genome sequence of *R. etli* is not yet available.

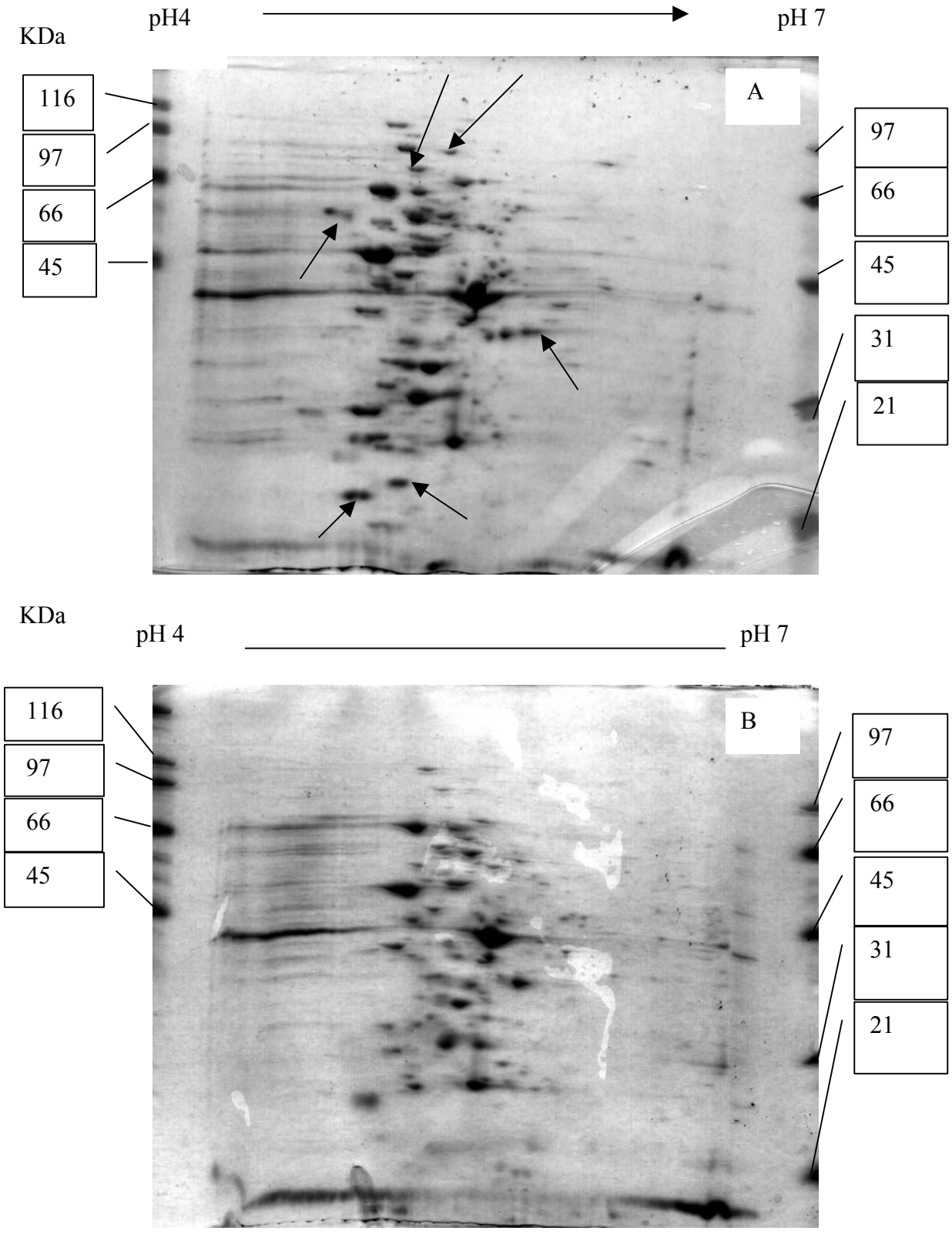

Figure 29. Comparison between the total soluble proteins of *R. etli* EBRI 26 under stress of salt 4% NaCl (A) and none induced (B) as a result of staining with CBB.

In the second method 50 µg of protein produced by strain EBRI 26 under standard conditions were labelled with Cy3 and 50 µg of protein from the same strain grown under salt stress, were labelled with Cy5 and mixed together. Fifty µg proteins of this mix was run for isoelectric focusing in immobilized pH gradient IPG (pH 4-7) and electrophoresed on SDS-PAGE for the second dimension as explained previously. Figure 30 show the different colours between proteins expressed under standard conditions with green colour and those produced under salt stress with red colour.

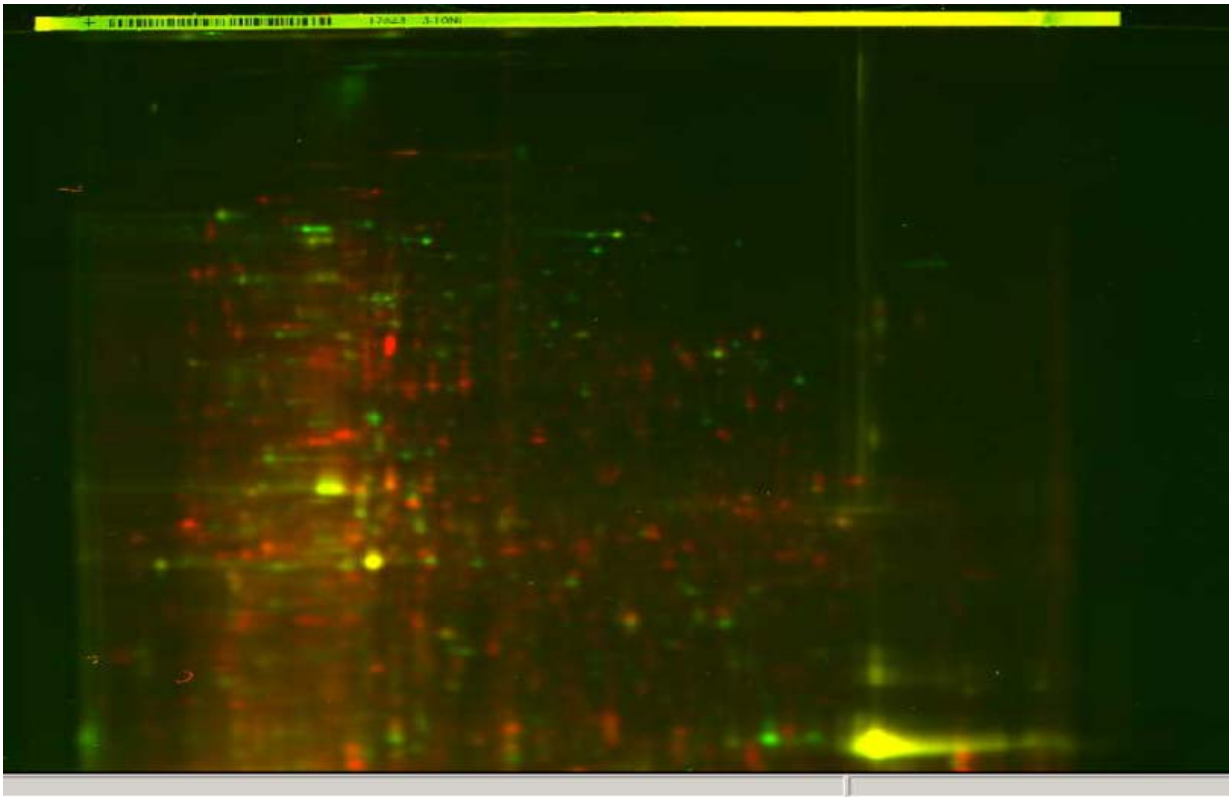

Figure 30. 2D-DIGE soluble proteins from strain EBRI 26 grown under standard conditions and labelled with Cy3 (green colour) compared to red spots produced under stress of 4% NaCl and labelled with Cy5.

Results in Table 21 summarize the data of proteins induced under salt stress and the control under standard conditions. Similarity percent between the proteins yielded under normal conditions and under salt stressed ranged from 94.6% to 95.6% for each replica. Using the computer software, 1119 spots of proteins

were analysed from replica 1 while 982 were analysed from replica 2. Thirty-five proteins decreased in their concentration and fourteen increased under salt stress in replica 1, while thirty-nine proteins decreased and fourteen increased in the second replication. The characteristics of proteins that over–expressed are summarized in Table A8.

Table 21. Summary of results from the experiment of 2D-DIGE labelling.

Characteristics	**Replica 1**	**Replica 2**
Spots included for comparison	1119	982
Number of similar proteins	1070	929
Percent of similar spots	95.6	94.6
Spots decreased	35	39
Spots increased	14	14

The method of DIGE labelling protein is more sensitive than the staining with CBB, probably due to the smaller amount of protein loaded (50 μg) compared to 700 μg with CBB staining.

3.8 Protein expression in *Sinorhizobium meliloti* strain 2011 after salt stress

All identified proteins have a high Mascot score except one and they have IPs from 4.84 to 6.11 (Table 22). Two groups of proteins were identified in *Sinorhizobium meliloti* strain 2011 under salt-stress (2.5% NaCl). The score of similarities and e-values are given in Table 22. The first protein was designated as a conserved hypothetical exported protein from the cellular periplasmic space belonging to a protein family of extracytoplasmic solute receptors. This protein could function as a receptor for osmotic solutes produced and over accumulated under salt stress. The second was designated as a probable carboxynospermidine decarboxylase. This protein is involved in biosynthesis of spermidine

(a polyamine) by decarboxylation of ornithine or arginine. The third protein was categorized as a conserved hypothetical protein, a member of amino acid transporters and metabolism. The fourth protein was designated as a probable isoleucyl-tRNA synthetase. This protein is related to a protein family of amino acid transferases and synthesis.

The second group contained six down regulated proteins (Figure 31b). The first one in this group is designated as an ABC transporters periplasmic solute binding protein, the second as catalase. The third protein is categorized as a citrate synthase. The fourth down regulated protein was designated as a O-succinylhomoserine sulfuhydrylase belong to biosynthesis of amino acids. The fifth one in this group was classified as a putative oxidoreductase protein with a function in cell envelope or outer membrane biogenesis. The last one was classified as 3-Hydroxybutyrate dehydrogenase. Ultimately, we can conclude that, the genes involved in salt tolerance are a set of genes which work together for osmoprotection.

Table 22. Identification of proteins affected under salt stress as over- expressed or down regulated from *Sinorhizobium meliloti* strain 2011.

Spot no.	Acc. no.	Protein identification	Mascot score	Score of similarity	E-value	Mw Da	IP	Sequence covering %
Over-expressed proteins								
1	G96020	Conserved hypothetical exported protein SMb20724	112	555	e-175	32271	4.84	35
2	F 96035	Probable carboxynospermidine decarboxylase	174	730	e-0.0	41292	5.35	53
3	E96035	Conserved hypothetical protein	129	810	e-0.0	45828	5.6	41
4	Q92RR0	Probable isoleucyl-tRNA synthetase	99	1983	e-0.0	109388	5.56	14
Down regulated proteins								
5	H95930	Hypothetical protein SM21133	186	642	e-0.0	37055	4.97	34
6	F95422	Catalase	275	1369	e-0.0	80299	5.6	39
7	CAC46088	SME591787	168	879	e-0.0	48427	6.02	35
8	Q92S95	Probable O-succinylhomoserine sulfuhydrylase	255	759	e-0.0	43074	5.78	62
9	Q92PA5	Putative oxidoreductase protein	153	422	e-117	25078	5.8	72
10	H95983	3-hydroxybutyrate dehydrogenase	54	506	e-142	27219	6.07	22

IP: Isoelectric point, nd: not determined.

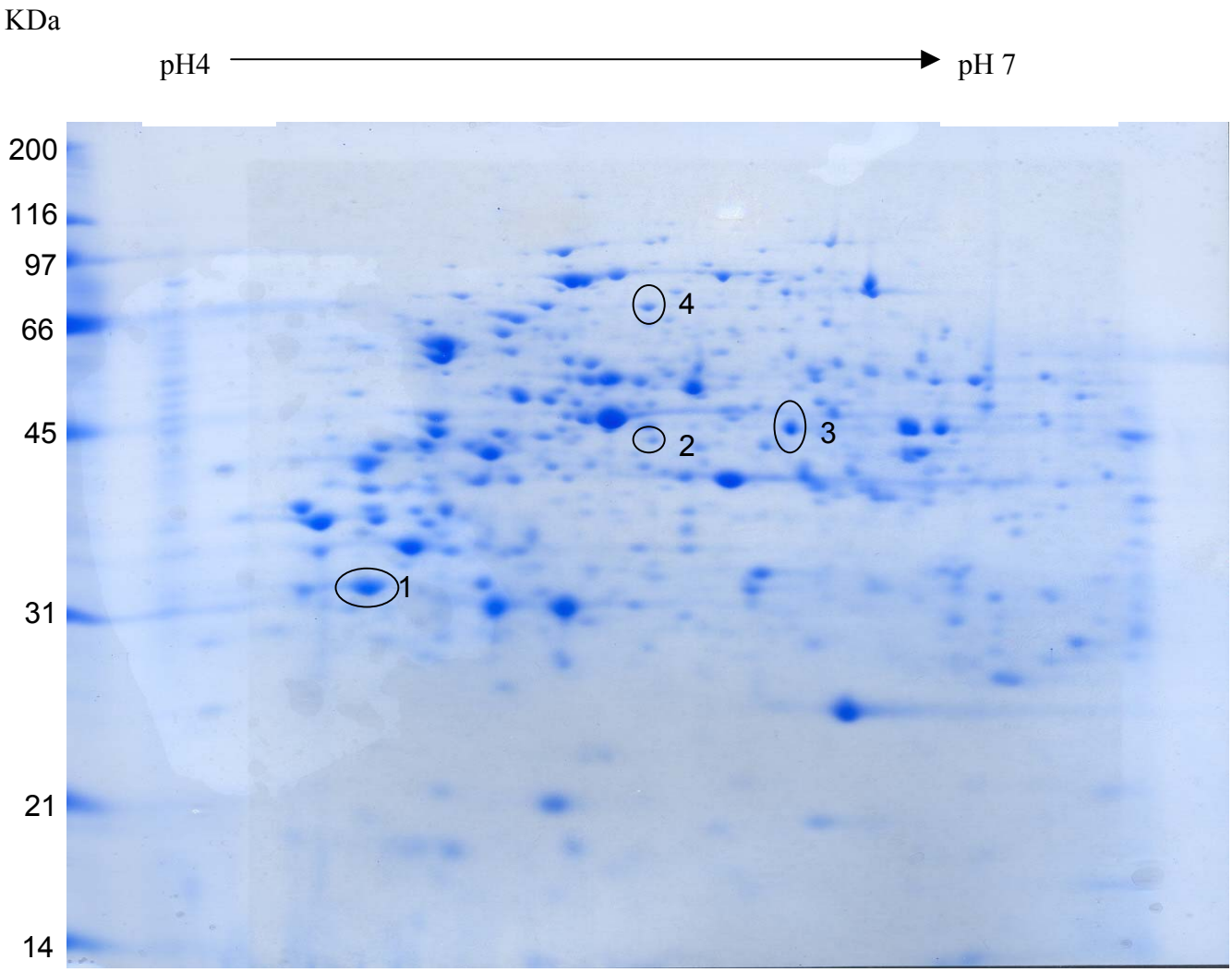

Figure 31a. 2D of soluble proteins from *Sinorhizobium meliloti* strain 2011 under stress of (2.5% NaCl), circled proteins from 1 to 4 are over expressed.

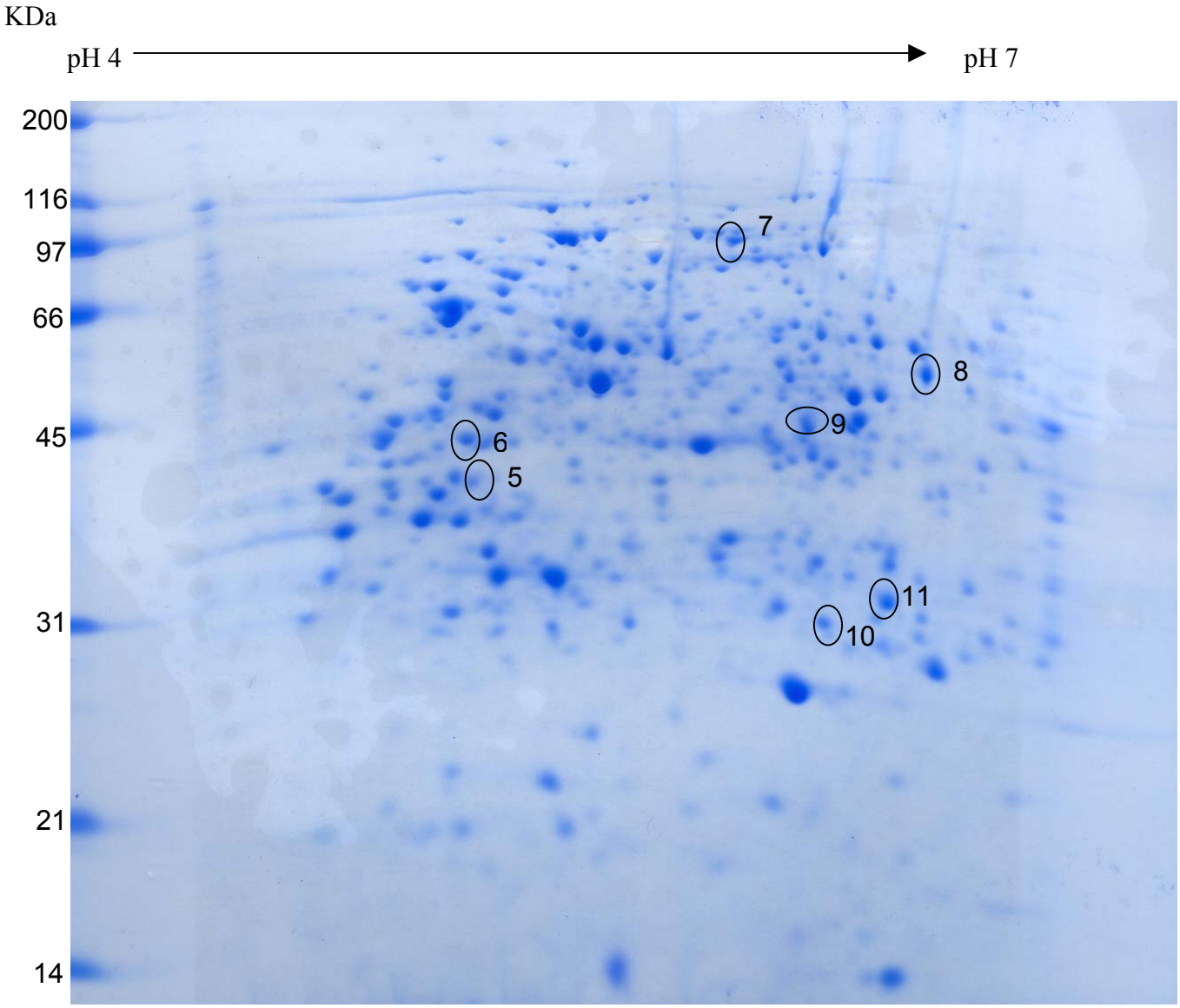

Figure 31b. 2D of soluble proteins from *Sinorhizobium meliloti* strain 2011 without stress of salt, circled proteins from 6 to 11 are down regulated .

4 Discussion

Phaseolus vulgaris can fix about 40-50% of their N-demand by symbiotic N_2 fixation (Werner, 1992), but efficiencies in the field are often much lower. This is partly due to the relatively broad microsymbiont specificity shown by common bean, which is known to nodulate with at least five rhizobial species with contrasting symbiotic competitiveness and efficiency (Martinez-Romero, 2003; Mhamdi et al., 1999; Silva et al., 1999 and 2003). Therefore it is important to select more effective strains for specific cultivars from the region where common beans are cultivated intensively. The genetic biodiversity of these strains using different molecular biology techniques and adaptation to environmental stresses must be studied.

4.1 Genetic biodiversity of bean rhizobial isolates and identification

To achieve the first goal, 12 strains were isolated from two geographical regions of Egypt to characterize them taxonomically as well as by their symbiotic phenotypes. The genetic biodiversity of the EBRI isolates was first analyzed by REP-PCR and amplified ribosomal DNA restriction analysis (ARDRA) using 16S and 23S rDNA. Rademaker et al. (1998) and Versalovic et al. (1994) have successfully used the highly reproducible and discriminative REP-PCR genomic fingerprint protocol in a wide variety of bacteria for typing strains and studying their diversity. Six different clusters were easily recognized among the twelve examined isolates based on REP-PCR fingerprint. The characterization of bean rhizobial strains by ARDRA can differentiate rhizobial strains nodulating common bean as shown by Sanatamaria et al. (1997) and Silva et al. (2003). A single amplification product of about 1500 bp was obtained from rhizobial strains using primers rD1 and fD1 while it was approximately 2100 bp using primers P3 and P4, indicating that 16S rDNA and 23S rDNA can be used for a wide range of *Rhizobium* strains (Weisburg et al., 1991 and Terefework et al.,

1998). It had been reported, that the ITS rDNA region of rhizobia is quite variable in length (Vinusea et al., 1998) which limits the extensive use of this marker in RFLP analysis for phylogenetic studies. Some of Egyptian bean rhizobial strains yielded two ITS amplification products of different size, findings similar to those obtained by Haukke et al. (1996) who found that three chromosomes may exist within a single rhizobial strain. Studying the genetic diversity of EBRI isolates by combining the ARDRA patterns from 16S and 23S rDNA revealed that most of the isolates were closely related to *Rhizobium etli*. Four strains were related to *Agrobacterium tumefaciens* (*Rhizobium radiobacter*), whereas only one isolate was related to *Rhizobium gallicum*. This was confirmed by phylogenetic analysis of nearly full-length 16S rDNA sequences, the key gene used in current rhizobial systematic for species identification (Sawada et al., 2003). The majority of EBRI isolates were in one clade with *R. etli* CFN 42. Strain EBRI 32 designated as *R. gallicum* in ARDRA results, also had nodulation characteristics and plasmid profile similar to *R. gallicum* R602sp but intermingled with the genetic lineage of *R. etli* CFN 42, suggesting a gene transfer causing the heterogeneity of rrs phylogeny of strain EBRI 32 (Ueda et al., 1999; Moulin et al., 2004). Similar results were reported by Herrera-Cervera et al. (1999) who found, that identical RFLP profiles of *nifH* and *nodB* genes were obtained from rhizobial isolates belonging to four different 16S rRNA gene species (*R. etli* bv. *phaseoli*, *R. leguminosarum*, *R. gallicum* and *R. giardinii*) explained by interspecific gene transfer. Also, Eardly et al. (1995) did show the occurrence of strains carrying the *R. leguminosarum* or *R. etli* 16S rRNA allele within closely related chromosomal lineage, suggesting a transfer or recombination of 16S rRNA gene as a possible explanation for their observation. On the other side, Fox et al. (1992) found that 16S rRNA sequence identity is not a sufficient method for species identification. Plamid analysis are more discriminative than the ARDRA method, within 16 rhizobial strain fourteen plasmid patterns were obtained. Our rhizobial strains contained from 2

to 6 plasmids, in agreement with the results from Thurman et al. (1985) who found that *Rhizobium* strains may contain from 1 to 10 plasmids. Plasmid profiles were in agreement with phenotypic characteristics, such as growth on LB medium, on basal medium with glucuronic acid as a main carbon source, on MM medium with malate or sorbitol as a carbon source, response to spectinomycin, colony morphology and nodulation parameters. Strains EBRI 2, 3, 21, 24, 26, 27, 29 and 32 were hybridized against a *nifH* probe to estimate the copy numbers of this gene. Strains EBRI 2, 3, 21, 24, 26, 27 and 29 had three copies of *nifH* gene. This supported the identification of these strains as *R. etli* bv. *phaseoli* (Segovia et al., 1993). Strain EBRI 32 had only one copy of *nifH* gene, confirming the identification of this strain as *R. gallicum* bv. *gallicum* (Amarger et al., 1997). Based on this polyphasic analysis, we conclude that the isolates analysed in this study correspond to *R. etli* bv. *phaseoli*, *R. gallicum* bv. *gallicum* and a *Rhizobium* lineage closely related to *R. radiobacter* (former *Agrobacterium tumefaciens*) as reported by Shamseldin et al. (2005). These results are consistent with those reported for common bean nodule isolates, obtained from Jordan (Tamimi and Young, 2004), from Tunisia (Mhamdi et al., 1999; 2002) from Central and West Africa (Diouf et al., 2000) and from Ethiopia (Beyene et al., 2004) who found that *R. etli* is the dominant common bean micro-symbiont in these soils. Although the last authors did not find *Agrobacterium*-like isolates, others have isolated such bacteria from diverse hosts, including common bean nodules from Morocco (Drevon et al., 2001), from tropical area (de Lajudie et al., 1999), from Tunisia (Mhamdi et al., 2002), from legume nodules from Pakistan (Hameed et al., 2004) and from Egyptian soils (Shamseldin et al., 2005). The *Agrobacterium*-like isolates seem to be symbiotically unstable under laboratory conditions and easily lose the ability to nodulate the host, which is likely due to loss of key symbiotic genes or the entire Sym-plasmids as evidenced by our *nodC* PCR amplification and plasmid profiling experiment. The symbiotic instability of *Agrobacterium*-like isolates

has been also observed by other workers (de Lajudie et al., 1999;Martinez et al., 1987) and this may be the most probable explanation for the loose of the symbiotic phenotype of isolates EBRI 6, 20, 23 and 25. Such isolates are therefore clearly a poor choice for the formulation of common bean inoculants. Hungria and Araujo (1995) reported, that the strain SEMIA 4064, used as a commercial inoculants lost its ability to fix nitrogen with common bean under field and green house conditions. This observation indicates that the inoculation programs should be directed not only to select effective strains but also to select strains with genetic stability to avoid the loss of the Sym plasmid or genomic rearrangements (Flores et al., 2000).

4.2 Efficiency and compatibility between plant host and rhizobial strains

The nodulation experiments with *P. vulgaris* cv. Saxa from Germany revealed, that the best symbiotic performance for shoot fresh weight and N_2 fixation was obtained with the *R. etli* strains EBRI 21, 2 and 26. These strains were selected to study their efficiency with two other plant cultivars, Canoca from Colombia and Giza 6 from Egypt. We found a remarkable degree of specificity for each strain. The best strains for nodulation of the cultivar Giza 6 were EBRI 2 and 21, and for cultivar Canoca were EBRI 21 and 26. On the other hand, the most effective strains for nitrogen fixation were CIAT 899 and EBRI 26 with Giza 6, while EBRI 2 and 26 were the most effective strain with cultivar Canoca. A high degree of specificity between microsymbionts and the host cultivars were also reported by Buttery et al. (1987 and 1990), Long, (1989) and Moawad et al. (2004). These strains were also tested against *Leucaena leucocephala* cv. Cunningham. Only one strain (EBRI 32) was able to nodulate this tree legume and failed to grow on LB medium, which is consistent with its classification as *R. gallicum* bv. *gallicum* (Amarger et al., 1997).

4.3 Selection of well adapted strains to environmental stresses

Ten percent of degraded soils in the world are high saline or high alkaline soils (Surange et al., 1997). Arid land in Egypt represents 97% of the total area, characterized by high temperature, low relative humidity, high rate of evaporation and little rainfall (Zahran, 1999). Consequently, we aimed to test the ability of our new strains to grow under different kinds of environmental stresses such as salinity, alkalinity, acidity, temperature and interaction between high salt and pH. Among eight *Rhizobium* strains from Egyptian soils, two physiological groups were identified based on the resistance or sensitive to salinity. The first group contained strains EBRI 21, 24, 26, 27 and 29, highly tolerant to salt stress of 3% or 4% NaCl (Shamseldin and Werner, 2005). Priefer et al. (2001) isolated *Phaseolus*-symbionts tolerant to 4% NaCl from Morocco. Our data are also consistent with results obtained by Elsheikh and Wood (1995) who found that some strains of fast growing-acid producing rhizobia such as *R. etli* were generally more salt tolerant than slow growing-alkaline producing strains. The second group included strains EBRI 2, 3 and 32, very sensitive to salt stress. The tolerance of rhizobial strains to salinity, alkalinity, acidity and temperature is more strain specific than species specific (Amarger et al., 1997). The standard strain *R. tropici* CIAT 899 is also able to resist NaCl concentration up to 2.5%, but it was able to grow on 4% NaCl when the incubation time was prolonged to six days (data not shown). Similar results have been reported by Nogales et al. (2002). Strains examined in this study were not only resistant to salt stress but were also tolerant to low and high pH. Under low pH, strain *R. tropici* CIAT 899 isolated from acid soils in Colombia was reported to be more tolerant to low pH (Steele et al., 2003; Vinuesa et al., 2003) than the Egyptian strains. A significant positive correlation was observed between salt tolerance and the adaptation of rhizobial strains to alkaline conditions (Kulkarni et al., 2000; Shamseldin and

Werner, 2005). Nodule function in *Phaseolus vulgaris* are limited by root temperature above 30 °C (Piha and Munns, 1987; Michiels et al., 1994).
Strains EBRI 2 and 26 were highly tolerant up to 42 °C. Karanja and Wood (1988) have isolated bean rhizobial strains which could even grow at 45 °C. In the interaction between salinity and alkalinity tolerance, the salt tolerant strains EBRI 21 and 26 were more adapted to alkaline pH than the salt sensitive strain EBRI 2. Similar results were obtained by Kulkarni et al. (2000).

4.4 Symbiotic performance of rhizobial strains under high salt or high pH

The effects of salt stress on nodulation and nitrogen fixation of legumes have been examined in several studies (Delgado et al., 1994; Moawad and Beck 1991; Elshinnawi et al., 1989; Igual et al., 1997; Nair et al., 1993; Subba Rao et al., 1990; Shamseldin and Wener, 2005). Strains EBRI 2 and EBRI 26 as salt sensitive and salt resistant respectively were examined to test their ability to nodulate and fix nitrogen under salt stress or high pH with cultivars Giza 6 and Saxa. In cultivar Giza 6, the symbiotic capacity of the salt sensitive strain EBRI 2 under low level of salt (0.2% NaCl) was equal with its ability at normal condition. At 0.4% NaCl, the formation of nodules by this strain was extremely affected as reported for other interactions (Bekki et al., 1987; Saadallah et al., 2001 and Cordovilla et al., 1999). The addition of different levels of sodium chloride enhanced the nodualtion with the salt tolerant strain EBRI 26. Our results confirmed also some results by Elsheikh and Wood (1995) with soybeans and some nodulating strains. With cultivar Saxa, the formation of nodules was not affected but nitrogen fixation was affected. Both strains, EBRI 2 and EBRI 26 were able to nodulate and fix nitrogen under high pH indicating that the detrimental effect of high pH was less severe than the stress of salinity. Surprisingly, nitrogen fixation per nodule fresh weight increased under high alkaline condition (pH8 and pH 8.5). Similar results have been reported by Evans et al. (1988) with increase in nodule formation in alkaline soils.

4.5 Selection of the most competitive and well adapted strains to environmental conditions in Egypt, using *gus* reporter gene

4.5.1 Marking of a *R. tropici* CIAT 899G with the *gus* gene

First, we tried to construct *Rhizobium etli* strain EBRI 26 with a *gus* gene but the transconjugants lost their ability to nodulate the host *Phaseolus vulagris* as compared with the parent strain (data not shown). This failure of the transconjugants to nodulate common bean was correlated with the insertion of transposon Tn5 which can form mutants affecting genes required for symbiosis (Anyango et al., 1998). Anyango et al. (1998) have observed the same problem with *R. etli* strain CFN42 and they explained this with a genetic instability in this species, known to have a high frequency of rearrangements in its genome (Flores et al., 2000). Based on this observation, strain *R. tropici* CIAT 899 was constructed with *gus* reporter as a stable strain CIAT 899G.

4.5.2 Competition between strain CIAT 899 wt and its derivative CIAT 899G

No statistically significant differences were detected in nodule number among plants inoculated with CIAT 899G or the parent strain. This revealed the suitability of CIAT 899G for a quantitative analysis of nodule occupancy in co-inoculation experiments, after proofing that this strain is stable and not significantly affected in its competitive ability due to the insertion of the reporter gene (Streit et al., 1995; Sessitsch et al., 1997; Shamseldin and Werner, 2004).

4.5.3 Selection of competitive strains under normal conditions

To estimate the nodule occupancy, roots with dual strain inoculation were incubated in a *gus* staining buffer to recognize the blue and white nodules. The staining of nodules was strong due to the plasmid mTn5ssgusA30 used,

containing the *nifH* promoter of *R. etli* strain CFN42 and an upstream activating sequence responsible for enhancing the activity of the *gus* gene in nodules (Wilson et al., 1995). Strains *R. etli* EBRI 2 and EBRI 26 were strong competitors against strain CIAT 899G only in the Egyptian cultivar Giza 6 with 66% and 67.5% nodule occupancy (Shamseldin and Werner, 2004). The results are in agreement with those obtained by George and Robert (1992) who found that *R. tropici* CIAT 899 was moderately competitive. Anyango et al. (1998) found that *R. tropici* CIAT 899 had only 14% of nodule occupancy when examined against *R. etli* Kim5 in a near-neutral pH soil, but formed 35% of nodules in acid soils. Moawad et al. (2004) found that the native rhizobial strains from Egyptian soils were more competitive and compatible with cultivar Giza 6 than foreign strains. A small percent of nodules (0.61-1.2%) were partially stained blue, indicating a double nodule occupancy, as observed by Krishnan and Pueppke (1992). Competitiveness indices were estimated using the equation described by Beattie et al. (1989). Records of competitiveness indices indicate that the two Egyptian rhizobial strains are highly competitive against CIAT 899G.

4.5.4 Selection of competitive strains under salt stress

Generally, salinity stress decreased nodule number, nodule fresh weight and shoot fresh weight as observed by Bekki et al. (1987) and Bordeleau and Prevost (1994). For estimating nodule occupancy under salt stress, it is interesting to note that, the salt sensitive strain EBRI 2 (tolerant only to 0.5% NaCl) was a better competitor with 87.4% of nodule occupancy than strain EBRI 26 (tolerant to 4% NaCl) with 63.7% of nodule occupancy against CIAT 899G (9.4% and 33.1%) at 0.2% NaCl (Shamseldin and Werner, 2004). Subba Rao et al. (1990) found that *Rhizobium* strains well adapted to form effective symbiosis with their hosts at saline conditions are not necessarily obtained from saline soils. Also, Nair et al. (1993) noticed that some rhizobial strains tolerating extremely high

levels of salt, had a significantly lower symbiotic efficiency under salt stress. This indicates that the competitiveness of strains under stress of salt is not only due to the ability of strains to resist the increasing level of sodium chloride but also to other factors such as motility and chemo taxis (Mellor et al., 1987; Zodor and Pueppke 1991), cell surface polysaccharides (Handelsman et al., 1984; Lagares et., 1992) and bacteriocin production (Triplett and Barta 1987).

4.5.5 Selection of competitive strains under alkalinity stress

In all cases, the high pH had no detrimental effect on nodulation (Evans et al., 1988) compared to stress of salinity. Although the strains EBRI 26 and CIAT 899G are more resistant to alkalinity in liquid culture (maximum level of growth pH 9) than strain EBRI 2 which was moderately tolerant to alkaline pH (maximum level of growth pH 8.5), strain EBRI 2 gave the highest percent of nodule occupancy 83% compared with EBRI 26 (53.2%) against CIAT 899G. There are many reports about the problems of nodulation and nitrogen fixation in acid soils (Lie, 1981; Graham et al., 1982; Wood et al., 1984, Graham, 1992; Bordeleau and Prevost, 1994 and Carter et al., 1994) but there are only a few studies about the same problem in alkaline soils.

4.5.6 Selection of competitive strains under temperature stress

High temperatures affected nodulation and nitrogen fixation as much as salinity-stress. A large reduction in nodule number and nodule fresh weight was observed at 35 $^{\circ}$C degree. These results are in agreement with those obtained by Michiels et al. (1994) with reduced nodulation in common bean at high soil temperature. Temperature stress affected not only nodule number and nodule sizes but also nodule functions. Similar results were obtained by Piha and Munns (1987) who noticed that nodule functions in common bean are optimal at 25 $^{\circ}$C or 30 $^{\circ}$C but are inhibited by root temperatures above 30 $^{\circ}$C. The nitrogen fixation capacity was strongly reduced at 35 $^{\circ}$C (Michiels et al., 1994). Although

strains EBRI 2 and EBRI 26 could survive under 42 °C in liquid culture, strain *R. etli* EBRI 2 was more competitive with 81.5% than *R. etli* EBRI 26 with 64.7% of nodule occupancy against *R. tropici* CIAT 899G at 35 °C. Strain EBRI 2 was also competitive under salinity and alkalinity. Silva et al. (2003) and Shamseldin and Werner (2004) found that *R. etli* is more competitive for *P. vulgaris* nodulation than other species.

4.6 Effect of nitrogen fertilizer application and rhizobial inoculation on nodulation of *Phaseolus vulgaris*

Application of low levels of potassium nitrate enhanced nitrogen fixation while high levels of potassium nitrate (80 ppm N) were inhibitors. A reduction in nodule number by 44% and 52% was obtained with strains EBRI 2 and EBRI 26 at 80 ppm N. Similar results were reported by Eaglesham (1989) and Danso et al. (1990).

4.7 Approaches of proteom analysis of *R. etli* strain EBRI 26 under salt stress

4.7.1 CBB staining and MALDI-TOF analysis

2D MALDI-TOF is a powerful and highly sensitive tool to analyse proteins especially when enough information from the Data-banks are available (Klose, 1999; Görg et al., 2000; Peick et al., 1999). *R. etli* strain EBRI 26, identified as salt tolerant (Shamseldin and Werner, 2005) was selected to study the proteins affected by salt stress. Samples of proteins were analysed using 2D technique and spots were detected using two methods. In the first method, spots of protein were recognized by staining in CBB dye after the second dimension. Six SIP (salt induced proteins) were easily recognized as over-expressed, after application of salt stress (4% NaCl) compared with the control cells. These proteins have IPs (isoelectric points) between 5 to 5.5 and their masses are approximately 22, 25, 40, 65, 70 and 95 KDa respectively. A protein with a relative mobility of 65 KDa expressed under salt stress in *Rhizobium* strain was

observed by Zahran et al. (1994). Also Zahran et al. (2004) detected a protein of about 40 KDa involved in salt tolerance in *Rhizobium leguminosarum* bv. *vicia*. Unni and Rao (2001) they detected an over expression in four proteins of about 22, 38, 68 and 97 kDa in *Rhizobium sp* (ST1) due to growth under salt stress. These spots were picked up, digested with trypsin and analysed with a MALDI-TOF mass spectrometry. We obtained a good MFP (mass finger print, data not shown) but we could not identify these proteins because the genome sequence of *R. etli* still is not published. Similar difficulties were observed by Encarnación et al. (2003) who found that the small number of sequences for *R. etli* from databank limited the identification of genes in this micro organism using, MALDI-TOF mass spectrometry.

4.7.2 Second approach of CyeDyes labelling

Different staining techniques are commonly used which differ in their sensitivity and compatibility with mass spectrometric MS analysis (Lauber et al., 2001; patton, 2002). Commassie Brilliant Blue (CBB) is one of the most used techniques (Klose, 1999). Recently, von Eggeling et al. (2001) and Gade et al. (2003) have established a new approach called 2D-DIGE, based on staining with CyDyes prior to the first dimension of gel electrophoresis in contrast with the previous method, in which the staining is carried out after the second dimension. Spots were detected by scanning the gel with a Typhoon scanner after the second dimension, thirty-five proteins decreased in their concentration and fourteen increased after salt stress in replica 1, while thirty-nine proteins decreased and fourteen increased in the second replication. The results correlate with those from Botsford (1990) who reported that 41 proteins as affected in salt-stressed cells of *Escherichia coli*.

4.8 A proteomic approach of *Sinorhizobium meliloti* strain 2011 under salt stress

Two groups of proteins were identified in *Sinorhizobium meliloti* strain 2011 under salt-stress, because there are much sequences available from databanks for this species with a completed genome project (Galibert et al., 2000; Galibert et al., 2001). The first group included four over expressed proteins under salt stress. The first protein was designated as a conserved hypothetical exported protein from cellular periplasmic space belonging to a protein family of extracytoplasmic solute receptors. This kind of protein functions as a receptor for osmotic solutes, produced and over accumulated by bacterial cells under salt-stress. Several investigators have reported the accumulation of organic solutes under salt stress (Rai et al., 1985; Oren, 1999; Gouffi et al., 1999; Csonka and Hanson, 1991; Smith et al., 1994; Wood et al., 2001). The second protein was classified as a probable carboxynospermidine decarboxylase. This protein is involved in biosynthesis of spermidine (polyamine) by decarboxylation of ornithine or arginine. These results are in agreement with those by Aziz et al. (1999) and Fujihara and Yoneyama (1993) who noted an increase of polyamines as osmoprotectants under salt stress. The protective function of polyamines is mainly due to their cationic nature at cellular pH at which they can bind proteins and lipids in order to stabilize cellular structures (Tiburico et al., 1994). Polyamines have also been proposed to act as radical scavengers (Drolet et al., 1986) or as regulators of K^+ channels (Liu et al., 2000). The carboxynospermidine enzyme has catalytic activity and act on ornithine and arginine. TeChien et al. (1992) noticed an increase of arginine in *Rhizobium leguminosarum* due to osmotic stress. The third protein is a member of amino acid transporters and metabolism. TeChien et al. (1992) reported the increase of amino acids production under salinity stress. The fourth protein is a probable isoleucyl-tRNA synthetase. This protein is involved in protein biosynthesis. Nogales et al. (2002) have identified a protein belonging to the same protein

family (aminoacyl-tRNA synthetase), involved in salt tolerance of *Rhizobium tropici* CIAT 899.

The second group contained six down regulated proteins under salt stress. The first protein was classified as a protein belonging to the ABC transporters periplasmic solute binding proteins. The second protein was classified as catalase. This result may be related to the observation of Cavalcanti et al. (2004) who reported, that catalase activity decreased significantly in leaves of cowpea under stress of salinity. Tejera et al. (2004) reported a reduction of catalase activity by 30 to 60% in nodules, formed by *R. tropici* on roots of *Phaseolus vulgaris* at 25 mM NaCl while this reduction in catalase activity reached 75-95% in nodules induced at 50 mM. The decrease in catalase activity would lead to an increase in hydrogen peroxide levels. The third down regulated protein was categorized as citrate synthetase, belong to a protein family of energy production and conversion. The slow down of energy production is a normal response of cells under different kinds of environmental stresses until adaptation has been achieved to resume the growth. The fourth down regulated protein was identified as an probable O-succinylhomoserine sulfuhydrylase related to biosynthesis of amino acids. Rüberg et al. (2003) noted that seven genes encoding amino acid biosynthesis were repressed in *Sinorhizobium meliloti* strain 1021 under salt stress. The fifth protein was classified as a putative oxidoreductase protein with a possible function in cell envelope or outer membrane biogenesis. This could be involved in the transportation of ions from and into the cytoplasm. Djordievic (2004) noticed, that putative oxidoreductase proteins were detected in bacteroids of *Sinorhizobium meliloti* but were not detected in free living cells. Bolaños et al. (2003) reported that salt stress produces nodule-degraded cell walls, dramatically altered in several zones. The last protein of this group is classified as 3-Hydroxybutyrate dehydrogenase. Poly-B-hydroxybutyrate is a bacterial intracellular carbon reserve accumulating in the bacteroid stage.

These results mean, that genes involved in salt tolerance are a set of genes, are over-expressed or down regulated to adapt rhizobial cells under these harsh conditions. However, we are still far a way to understand, how these very different functions are linked together in a signal pathway of salt stress adaptation.

5 Summary

To maximize the benefits of symbiotic nitrogen fixation with common bean in Egyptian agriculture, twelve isolates of bean rhizobia were isolated from two geographical regions, where *Phaseolus vulgaris* is repeatedly cultivated. The efficiency of strains was examined with the German cultivar Saxa. Results revealed that eight isolates were able to form nodules on the roots of common bean host Saxa but four were unable to form nodules on this host. Isolate EBRI 29 was the best strain for nodule numbers (129 nodules plant^{-1}) and biomass (907 mg fresh weight nodules plant^{-1}) while strains EBRI 2, 21 and 26 were the most effective strains for specific nitrogen fixation (8.9, 13.7 and 7.9 n mol $C_2H_4h^{-1}$ mg^{-1} nodules) respectively. Based on these results, strains EBRI 2, 21 and 26 were selected to study the host specificity compared with the standard type strain of *R. tropici* CIAT 899. The cultivars Saxa, Giza 6 and Canoca from Germany, Egypt and Colombia were used for this purpose. Results indicate, that there is a high degree of host specificity. Strains EBRI 2, 21 and 26 from Egypt were the most effective strains for nodule numbers, nodule fresh weight, shoot fresh weight and nitrogen fixation with cultivar Giza 6, while the Colombian strain CIAT 899 was the best strain with cultivar Canoca from South America.

Isolates were characterized using molecular biology methods such as REP-PCR, amplified rDNA restriction analysis (ARDRA) of 16S rDNA, 23S rDNA and 16S-23S rDNA (Internal transcribed spacer), plasmid profile analysis, hybridisation with *nifH* probe, amplification of *nodC* gene and sequences of full length 16S rDNA. Six different clones were identified among the isolates based on REP-PCR. The isolates and standard strains *R. etli* CFN42, *R. tropici* CIAT 899, *R. gallicum* R602sp, *R. giardinii* H152 and *A. tumefaciens* 30150 DSM gave amplified fragment of 1500 bp of the rrs locus and a fragment of 2100 bp of the rrl amplicons. The ITS (Internal transcribed spacers) between 16S and 23S rDNA could not be used because some of the examined strains

produced two amplified fragments, different in their size. Products from 16S rDNA were digested with restriction enzymes *HhaI*, *DdeI*, *MspI* and *Sau3aI* while the products from 23S rDNA were digested with enzymes *HinfI*, *MspI* and *Sau3aI*. A combination between the restriction analysis of rrs locus and rrl amplicons indicated, that three genotypic groups were identified among the twelve strains. Genotype A included strains EBRI 2, 3, 21, 24, 26, 27 and 29 belonging to *Rhizobium etli*. Genotype B contained only strain EBRI 32, associated with the cluster of *Rhizobium gallicum*. Genotype C included *Agrobacterium*-like isolates EBRI 6, 20, 23 and 25, unable to re-nodulate common bean and sharing the cluster with *Agrobacterium tumefaciens*.

Plasmid profile analysis revealed a higher genetic diversity than REP-PCR and ARDRA techniques. Among the 16 rhizobial strains examined, fourteen plasmid patterns were found. Plasmid numbers varied from 2 to 6 and their sizes ranged from 40 kb to 650 kb. Only one mega plasmid was detected from strains *R. tropici* CIAT 899 and *R. etli* EBRI 29 but not resolved from *R. gallicum* R602sp and EBRI 32. Strains EBRI 2, 3, 21, 24, 26, 27, 29 and 32 yielded amplified fragment of about 1450 bp of *nodC*, consistent with their classification as symbiotic isolates, while strains EBRI 6, 20, 23 and 25 did not give amplified fragments of *nodC*, consistent with their classification as non-symbiotic isolates. Strains EBRI 2, 3, 24, 26, 29, 32 and CIAT 899 produced two amplified fragments of the *nodC* gene, indicating that they have two copies but the other strains had only one fragment. Results from 16S rDNA sequences confirmed those obtained from ARDRA results for all strains except strain EBRI 32, clustering with *R. gallicum* R602sp in the ARDRA results but associated to the lineage of *R. etli* CFN 42 in the phylogentic tree. Symbiotic isolates EBRI 2, 3, 21, 26 and 27 formed a highly supported clade with *R. etli* CFN 42. Non symbiotic isolates EBRI 6, 20, 23 and 25 were significantly closest to the lineages of *R. radiobacter* and *R. rubi* previously named *Agrobacterium radiobacter* and *Agrobacterium rubi*. We can conclude that, the isolates used in

this study belong to *R. etli*, *R. gallicum* and a *Rhizobium* cluster closely related to *R. radiobacter* (former *Agrobacterium tumefaciens*). Hybridization with the *nifH* probe supported the results of 16S rDNA sequences. Strains EBRI 2, 3, 21, 24, 26, 27 and 29 had three copies of *nifH* gene consistent with their classification as *R. etli* biovar *phaseoli*. Strain EBRI 32 had only one copy of *nifH* gene in agreement with its identification as *R. gallicum* biovar *gallicum*. Initial nodulation experiments with *Phaseolus vulgaris* and *Leuceana leucocephala* also supported this identification due to the ability of strain EBRI 32 to nodulate both hosts, while *R. etli* strains were only able to form nodules on *P. vulgaris*. Additional physiological experiments were done to confirm these results. All *R. etli* strains were able to use malate as a main carbon source but not sorbitol, growing on MM medium and they failed also to grow on LB medium. *Agrobacterium*-like isolates were able to grow on LB medium. Strain EBRI 32 was not able to grow on basal medium containing glucuronic acid as a sole carbon source or with 5 μg ml^{-1} spectinomycin, and was not able to grow on LB medium as previously reported for *R. etli*, *A. tumefaciens* and *R. gallicum*.

An important objective was to select the best adapted strains under environmental stresses and to study their symbiotic performances under these conditions. Strains were classified into two groups due to their tolerance or sensitivity to salt stress. The first group included strains *R. etli* EBRI 21 and EBRI 26 resistant to sodium chloride up to 4%, and strains *R. etli* EBRI 24, 27 and 29 able to survive under 3% NaCl. The second group was salt sensitive, including the two *R. etli* strains EBRI 2 and EBRI 3, and *R. gallicum* EBRI 32. They survived only at 0.5% NaCl or less. Strains examined in this study were tolerant also to high soil pH. *R. etli* strains isolated from alkaline soil in Egypt were less tolerant to low pH than the acid resistant strain *R. tropici* CIAT 899 isolated from acid soil in Colombia. The majority of strains were able to survive under 37 °C, while *R. etli* strains EBRI 2 and EBRI 26 did even grow at 42 °C.

R. etli strains isolated from alkaline soils in Egypt, are better adapted to the interaction between stress of salt and high pH than strain *R. tropici* CIAT 899. The specific nitrogenase activity of the salt tolerant strain EBRI 26 with cultivar Giza 6 was better than that of the salt sensitive strain EBRI 2, with 18.2 compared to 13.2 n mol C_2H_4 h^{-1} mg^{-1} nodule fresh weight. With the bean cultivar Saxa, nitrogen fixation was much more affected by a salt concentration of 0.4% NaCl with both salt tolerant and salt sensitive strains (3.9 and 3.8 n mol C_2H_4 h^{-1} mg^{-1} nodule fresh weight). No detrimental effect was observed on nodulation and nitrogen fixation under alkalinity stress.

Our goals were also directed to select the most competitive strains under normal or stress conditions. Strain *R. tropici* CIAT 899 was constructed with a *gus* reporter gene due to its genetic stability to estimate the percentages of nodule occupancy at a 1:1 ratio inoculation. Strain *R. tropici* CIAT 899G with the *gus* gene was tested against *R. etli* strains EBRI 2 and EBRI 26 with *Phaseolus* cultivars Giza 6 and Saxa. At neutral pH, the two *R. etli* strains EBRI 2 and EBRI 26 were more competitive than *R. tropici* CIAT 899G with both cultivars. They gave nodule occupancies of 52.1% and 61.1% with cultivar Saxa,, but nodule occupancies increased to 66% and 67.5% with the bean cultivar Giza 6 from Egypt. Based on these results, cultivar Giza 6 was used to select the most competitive strains under stresses of salinity or alkalinity. Under stress of salinity 0.2% NaCl (34.2 mM NaCl), the salt sensitive strain EBRI 2 was more competitive than the salt resistant strain EBRI 26. Strain EBRI 2 gave 87.4% while strain EBRI 26 gave 63.7% nodule occupancy against CIAT 899G. The same trend of results was observed under stress of alkalinity (pH 8) and high temperature (35 $^{\circ}$C). Strain EBRI 2 gave nodule occupancy of 83% under alkaline conditions and 81.5% nodule occupancy under high temperature, compared to EBRI 26 occupied 53.2% and 64.7% of nodules competing with CIAT 899G.

The effect of nitrogen fertilizer on the symbiotic efficiency of rhizobial strains *R. etli* EBRI 2 and EBRI 26 was tested under different levels of potassium nitrate. Under low levels of nitrogen fertilizer (20 ppm N), a significant reduction in nodule number and nodule fresh weight was observed with both strains, but an increase was observed in shoot fresh weight and acetylene reduction. Nodulation was totally suppressed by addition of 120 ppm N. Nitrogenase activity decreased gradually with the addition of nitrate higher than 20 ppm N.

The last goal of this thesis was to study the protein expression involved in salt tolerance. Strain *R. etli* bv. *phaseoli* EBRI 26 identified as salt tolerant, was selected as a model strain to analyse the proteins involved in salt tolerance using two approaches of 2-D gel electrophoresis. In the first approach, the spots of proteins were detected by staining with CBB (Commassie Brilliant blue). After staining the gels with CBB, the spots of proteins were compared from cells under salt stress and from control cultures. Six spots of SIP (salt induced proteins) were easily recognized as highly over-expressed under stress of 4% NaCl compared to the control cells. These proteins have IP (Isoelectric Points) around 5 to 5.5 and masses of approximately 22, 25, 40, 65, 70 and 95 KDa. These spots were picked up and digested with trypsin and analysed using MALDI-TOF-MS. Although, we obtained a good MFP (Mass Finger Print), we could not identify these proteins, further because the genome sequence of *R. etli* is not yet published. In the second method the proteins were detected using a Typhoon scanning system after labelling the proteins with CyDyes and applying the method of DIGE. The proteins obtained from salt culture were labelled with Cy5 and the proteins obtained from control cultures were labelled with Cy3. An equal amount of each sample (50 μg protein ml^{-1}) was mixed and used for the first and second dimension as explained previously. After the second dimension, the gel was scanned directly using the Typhoon scanner at two wave lengths. The results were analysed using a computer software, concluding that forty-nine

proteins were affected by salt stress. Fourteen were increased in their concentration and thirty-five were decreased. The method of DIGE labelling protein was more sensitive and reproducible than the staining with CBB due to the smaller amount of protein loaded (50 μg) compared with 700 μg by staining with CBB.

Using the same technique of 2D-MALDI-TOF mass spectrometry we could identify two groups of proteins from *Sinorhizobium meliloti* strain 2011 due to the available full genome sequence. The first group contained four over-expressed proteins and the second group included six down regulated proteins. The most important one from the first group was designated as a probable carboxynospermidine decarboxylase, with an important role in the biosynthesis of spermidine (polyamine) as osmoprotectant. Two other proteins were increased, belonging to proteins involved in amino acid metabolism and extracytoplasmic solute receptors. The last protein in this group was categorized as isoleucyl-tRNA synthetase. The most important from the second group was classified as catalase. The reduction in catalase activity would lead to an increase in hydrogen peroxide (H_2O_2) levels. Proteins involved in energy production and conversion such as citrate synthetase were also down regulated. The reduction of energy production is a usual reaction under environmental stress like salinity until adapting themselves to continue growth. Proteins categorized as ABC transporters, putative oxidoreductase, O-succinylhomoserine sulfuhydrylase and 3-Hydroxybutyrate dehydrogenas belong to different areas of metabolism and biosynthesis. We can conclude that, proteins affected by salt stress are not part of a single regulon.

6 Zusammenfassung

Um die Nutzung der symbiotischen Stickstoffgewinnung für die Landwirtschaft in Ägypten durch Bohnen (*Phaseolus vulgaris*) zu maximieren, wurden zwölf verschiedene Bohnen nodulierende *Rhizobium*-Stämme isoliert. Diese Isolate stammen aus zwei geographisch unterschiedlichen Regionen in Ägypten, wo *Phaseolus vulgaris* dauernd kultiviert werden. Die Nodulationsfähigkeit dieser Isolate wurde mit der deutschen *Phaseolus vulgaris* Sorte „Saxa“ geprüft. Die Versuche zeigten, dass acht der 12 Isolate effektive Knöllchen bildeten. Bei vier Isolaten wurde keine Knöllchenbildung nachgewiesen. Die Auswertung ergab, dass die Isolate EBRI 2, 21 und 26 die effektivsten Stickstofffixierungsraten aufwiesen. Die höchste Knöllchenzahl und die größte Biomasse ergaben sich bei der Infektion mit Isolat EBRI 29. Auf Grund dieser Ergebnisse wurden die Isolate EBRI 2, 21 und 26 für Studien zur Wirtsspezifität ausgewählt. Als Vergleichsstamm wurde *Rhizobium tropici* CIAT 899 eingesetzt. Als Pflanzensorten wurden „Saxa“ aus Deutschland, „Giza 6“ aus Ägypten und „Canoca“ aus Kolumbien verwandt. Die Ergebnisse zeigten einen hohen Grad an endemischer Wirtsspezifität. Isolate aus ägyptischer Erde hatten mit der ägyptischen Sorte *Phaseolus vulgaris* Giza 6 die besten Ergebnisse in Stickstofffixierung, Knöllchenanzahl und Knöllchengewicht, der aus Kolumbien stammende *R. tropici* CIAT 899 Stamm mit der *Phaseolus vulgaris* Canoca, aus Kolumbien.

Die Isolate wurden mit Hilfe molekulargenetischer Methoden wie REP-PCR, Restriktions-Analyse (ARDRA) der 16S rDNA, 23S rDNA und 16S-23S rDNA, Plasmidprofilanalyse, *nifH*-Hybridisierung, *nodC*-Amplifizierung und Sequenzierung der kompletten 16S rDNA analysiert. Sechs unterschiedliche Klone wurden aufgrund der Daten der REP-PCR identifiziert. Innerhalb dieser Isolate gibt es keine Beziehung zwischen der Gruppierung und dem

Isolationsort. Die Isolate und die Standard Stämme *R. etli* CFN42, *R. tropici* CIAT 899, *R. gallicum* R602sp, *R. giardinii* H152 und *A. tumefaciens* 30150 DSM besaßen in der rrs Region ein Fragment von 1500 Basenpaaren, während das Fragment des rrl Amplicons 2100 Basenpaare groß war. Die ITS(Internal Transcribed Spacer) Region zwischen 16S und 23S rDNA war uneinheitlich, einige der überprüfen Stämme amplifizerten zwei Fragmente unterschiedlicher Größe. Die Produkte der 16S rDNA wurden mit den Restriktionsenzymen *HhaI*, *DdeI*, *MspI* und *Sau3aI* und die Produkte der 23S rDNA mit *HinfI*, *MspI* und *Sau3AI* verdaut. Die Kombination der Ergebnisse aus den Restriktionsanalysen des rrs Locus und des rrl Amplicons ergab drei genotypische Gruppen innerhalb der 12 Stämme. Genotype A umfasst die Stämme EBRI 2, 3, 21, 24, 26, 27 und 29, sie sind genetisch eng verwandt mit *R. etli* CFN 42. Genotype B enthält lediglich Stamm EBRI 32, verwandt mit *R. gallicum* R602sp. Zu Genotyp C gehören die *Agrobacterium* ähnlichen Isolate EBRI 6, 20, 23 und 25. Mit diesen Isolaten war es erwartungsgemäß nicht möglich, *Phaseolus vulgaris* zu nodulieren. Die Plasmidprofileanalyse ergab eine höhere genetische Diversität als die REP-PCR und ARDRA-Analyse. In den 16 überprüften Rhizobiumstämmen wurden 14 verschiedene Plasmidmuster gefunden. Die Plasmidanzahl variierte zwischen zwei und sechs und die Plasmidgröße zwischen 40 kb und 650 kb. Bei den Stämmen *R. tropici* CIAT 899 und *R. etli* EBRI 29 wurde nur ein Megaplasmid detektiert, bei den Stämmen *R. gallicum* R602sp und EBRI 32 konnte dieses nicht nachgewiesen werden. Die Stämme EBRI 2, 3, 21, 24, 26, 27, 29 und 32 besaßen ein 1450 bp großes *nodC* Fragment, das die Klassifizierung als symbiotische Isolate bestätigt. Die Stämme EBRI 6, 20, 23 und 25 hatten kein *nodC* Fragment, wodurch sie als nicht symbiotische Stämme charakterisiert wurden. Die Stämme EBRI 2, 3, 24, 26, 29, 32 und CIAT 899 zeigten zwei Kopien von *nodC* Fragmenten, während die übrigen Stämme lediglich ein *nodC* Fragment enthielten. Die Ergebnisse der 16S rDNA Sequenzanalyse bestätigen die ARDRA Ergebnisse, ausgenommen

Stamm EBRI 32. Dieser wird in der ARDRA-Anlayse in einem Cluster mit *R. gallicum* R602sp zusammengefasst und in der phylogenetischen Analyse mit *R. etli* CFN 42 verbunden. Die symbiotischen Isolate EBRI 2, 3, 21, 26 und 27 zeigen eine sehr enge Verwandtschaft mit *Rhizobium etli* CFN 42. Die nicht symbiotischen Isolate EBRI 6, 20, 23 und 25 waren den Stämmen *Rhizobium radiobacter* und *Rhizobium rubi* phylogenetisch am nächsten verwandt. Diese Stämme wurden bisher als *Agrobacterium radiobacter* und *Agrobacterium rubi* bezeichnet.

Die in dieser Studie gewonnen Isolate sind mit den Bakterienstämmen *R. etli*, *R. gallicum* identisch und mit *R. radiobacter* (ehemals *Agrobacterium tumefaciens*) nah verwandt. Die Ergebnisse der *nifH* Hybridisierung bestätigten die Ergebnisse der 16S rDNA-Sequenzanalyse. Die Stämme EBRI 2, 3, 21, 24, 26, 27, und 29 besitzen drei Kopien des *nifH* Gens übereinstimmend mit *R. etli* biovar *phaseoli*. Der Stamm EBRI 32 hat eine Kopie des *nifH* Gens und ist damit identisch mit *R. gallicum* biovar *gallicum*. Nodulationsexperimente mit *Phaseolus vulgaris* und *Leucaena leucocephala* bestätigten diese Identifikation. Nur Stamm EBRI 32 war in der Lage beide Wirtspflanzenarten zu infizieren, während bei den Infektionen mit *R. etli* Stämmen nur bei *P. vulgaris* eine Knöllchenbildung zu beobachten war. Um diese Ergebnisse zu untermauern wurden weitere physiologische Experimente durchgeführt. Von allen *R. etli* Stämmen konnten Malat, nicht aber Sorbit metabolisiert werden. *R. etli* Stämme wuchsen auf MM-Medium, nicht aber auf LB-Medium. *Agrobacterium* ähnliche Isolate waren imstande auf LB-Medium zu wachsen. Stamm EBRI 32 konnte auf einem Basismedium mit Glucuronsäure als C-Quelle oder auf einem Basismedium mit 5 µg ml^{-1} Spectinomycin angezogen werden, während er im Gegensatz zu *R. etli*, *A. tumefaciens* und *R. gallicum* auf LB-Medium kein Wachstum zeigte.

Ein wichtiger Untersuchungsbereich waren die Adaption der Stämme an Umweltstress und Untersuchungen zu den symbiotischen Leistungen unter

diesen Bedingungen. Die Stämme wurden aufgrund ihrer Toleranz oder ihres Stressverhaltens gegen Salz in zwei Gruppen geteilt. Die erste Gruppe waren die salztoleranten Stämme EBRI 21 und EBRI 26, sie waren gegen Natriumchlorid Konzentrationen bis 4% unempfindlich. Die Stämme EBRI 24, 27 und 29 konnten bei NaCl-Konzentrationen bis 3% NaCl überleben. Die zweite Gruppe *R. etli* EBRI 2, EBRI 3 und *R. gallicum* EBRI 32 war salzsensitiv. Sie überlebten bei Salzkonzentrationen unter 0,6%. Die in dieser Studie untersuchten Stämme zeigten ebenfalls eine große Toleranz gegenüber hohen pH-Werten im Boden. *R. etli* Stämme, die aus ägyptischen Böden mit basischem pH-Wert isoliert wurden, waren gegenüber niedrigen pH-Werten deutlich sensitiver als der Stamm *R. tropici* CIAT 899, der aus einem Boden in Kolumbien mit niedrigem pH-Wert isoliert wurde. Die Mehrheit der Stämme wuchs nicht mehr bei einer Temperatur von über 37°C, nur die Stämme *R. etli* EBRI 2 und EBRI 26 zeigten noch gutes Wachstum bei einer Temperatur von 42°C. Die aus ägyptischen Böden isolierten *R. etli* Stämme scheinen an die Interaktion zwischen Salzstress und hohem pH-Wert besser angepasst zu sein als der kolumbianische *R. tropici* CIAT 899 Stamm. Die symbiotische Leistungsfähigkeit des salztoleranten Stammes EBRI 26 mit der ägyptischen Wirtspflanze *Phaseolus vulgaris* Giza 6 war mit 18,2 nM C_2H_4 h^{-1} mg^{-1} Knöllchenfrischgewicht höher als die Effizienz des salzsensitiven Stammes EBRI 2 mit 13,9 nM C_2H_4 h^{-1} mg^{-1} Knöllchenfrischgewicht. In der Interaktion mit *Phaseolus vulgaris* Saxa wurde die Stickstofffixierungsrate von salztoleranten und salzsensitiven Stämmen durch eine erhöhte Salzkonzentration (0,4% NaCl) sehr viel stärker beeinflusst. Die Stickstofffixierungsraten lagen nur bei 3,8 bzw. 3,9 nM C_2H_4 h^{-1} mg^{-1} Knöllchenfrischgewicht. Unter alkalischen Stressbedingungen gab es keinen deutlichen Effekt bei der Knöllchenanzahl oder Stickstofffixierungsrate.

Ein weiteres Ziel dieser Arbeit war, die konkurrenzfähigsten Stämme sowohl unter Normalbedingungen, als auch unter Umweltstressbedingungen zu

selektieren. Bei Infektionen mit dem genetisch stabilen Stamm CIAT 899G (enthält ein *Gus* Gen) wurde eine Blaufärbung der Knöllchen, als Ergebnis der Inkubation mit dem Substrat x-gluc erreicht. Somit konnte bei der Knöllchenbesiedlung in Gegenwart zu testender Stämme leicht erkannt werden, welche Knöllchen durch Infektion mit CIAT 899G gebildet wurden. Bei der Infektion von *Phaseolus vulgaris* Giza 6 und *Phaseolus vulgaris* Saxa mit den Stämmen *R. tropici* CIAT 899G und den Stämmen *R. etli* EBRI 2 und EBRI 26 zeigte sich, dass unter pH-neutralen Bedingungen die beiden *R. etli* Stämme mit beiden *Phaseolus vulgaris* Sorten konkurrenzfähiger waren. Es ergaben sich Knöllchenbesiedlungsraten von 52,1% und 61,1% bei der Sorte Saxa. Bei Infektion der ägyptischen Sorte Giza 6 mit EBRI 2 und EBRI 26 stiegen diese Besiedlungsraten auf 66% und 67,5% an. Basierend auf diesen Ergebnissen wurde die ägyptische Sorte Giza 6 ausgewählt, sowohl unter Salzstress als auch unter Alkalisstress die konkurrenzfähigsten Stämme zu selektieren. Der salzsensitive Stamm EBRI 2 war unter Salzstress (0,2% NaCl, 34,2 mM) kompetitiver als der salzresistente Stamm EBRI 26. Stamm EBRI 2 hatte eine Knöllchenbesiedlungsrate von 87,4%, Stamm EBRI 26 hatte 63,7% in Konkurrenz zu CIAT 899G.

Der gleiche Trend zeigte sich unter Alkalistress (pH 8) und erhöhter Wachstumstemperatur (35°C). Bei Stamm EBRI 2 ergab sich eine Besiedlungsrate von 83% unter Alkalistress und 81,5% bei erhöhter Wachstumstemperatur im Vergleich zu EBRI 26 mit 53,2% und 64,7% in Konkurrenz mit CIAT 899G.

Der Einfluss von Stickstoffdüngung auf die symbiotische Effizienz von *R. etli* EBRI 2 und EBRI 26 wurde mit verschiedenen Konzentrationen von Kaliumnitrat getestet. Bei einem niedrigen Gehalt von Stickstoffdünger (20 ppm N) wurde bei beiden Stämmen eine niedrige Knöllchenanzahl und ein niedriges Knöllchengewicht festgestellt, die Stickstofffixierungsrate und das Sprossgewicht hingegen stiegen an. Die Knöllchenbildung wurde bei einer

Zugabe von 120 ppm N vollkommen unterdrückt. Die Stickstofffixierungsrate wurde durch Zugabe von mehr als 20 ppm Kaliumnitrat allmählich reduziert.

Die letzte Aufgabe dieser Studie war es die spezifische Proteinexpression salztoleranter Stämme zu untersuchen. Der salztolerante Stamm *R. etli* bv. *Phaseoli* EBRI 26 wurde als Modellstamm für die Analyse mittels 2D-Gelelektrophorese ausgewählt. Im ersten Schritt wurden hierbei die Proteinspots durch Färbung mit CBB (Commassie Brilliant Blue) detektiert. Nach dem Färben des Gels mit CBB konnten beim Vergleich der Stämme, angezogen unter Salzstress (4% NaCl), mit den nicht unter Salzstress angezogenen Stämmen sechs unterschiedlichen, nur unter Salzstress vorhandene Proteine erkannt werden. Diese Proteine hatten einen IP (Isolelektrischen Punkt) zwischen pH 5 und pH 5,5 und eine Masse von ungefähr 22, 25, 40, 65, 70 und 95 KDa. Diese Proteinspots wurden aus dem Gel isoliert, mit Trypsin verdaut und mit MALDI-TOF analysiert. Obwohl das Ergebnis des MFP (Mass Finger Print) klar war, konnten diese Proteine nicht weiter identifiziert werden, weil die Sequenz des Genoms bisher noch nicht publiziert wurde.

Mittels einer weiteren Methode wurden die Proteine mit dem Typhoon-Scanning System detektiert. Dazu wurden die isolierten Proteine der Kulturen aus Medien mit erhöhtem Salzgehalt mit den Fluoreszenzmarkern Cy3 und den unter normalen Bedingungen angezogenen Stämme mit Cy5 markiert, gemischt und auf einem 2-D Gel aufgetrennt. Danach wurde das Gel direkt mittels Typhoon-Scanner bei zwei verschiedenen Wellenlängen analysiert. Die Computerauswertung zeigte, dass durch Salzstress 49 Proteine beeinflusst wurden. Der Gehalt von 14 Proteinen erhöhte sich und bei 35 Proteinen nahm die Konzentration ab. Die Methode der Proteinmarkierung mit DIGE war sensitiver und reproduzierbarer als die Färbung mit CBB.

Mit der gleichen 2D-MALDI-TOF Methode konnten zwei Proteingruppen aus *Sinorhizobium meliloti* 2011 identifiziert werden. Gruppe eins umfasst, bei hoher Salzkonzentration, vier überexprimierte Proteine, Gruppe zwei beinhaltet

sechs Proteine, die unter hoher Salzkonzentration vermindert exprimiert wurden. Das wichtigste Protein aus Gruppe eins wurde als Carboxynospermidin-Decarboxylase, die eine wichtige Rolle in der Biosynthese von Spermidin (Polyamin) als Osmoprotektant spielt, identifiziert. Zu dieser Gruppe gehört auch ein induziertes, konserviertes hypothetisches Protein, das am Aminosäuremetabolismus beteiligt ist. Ein weiteres konserviertes hypothetisches Protein gehört zu den „extracytoplasmic solute rezeptors“. Das letzte Protein dieser Gruppe wurde als Isoleucyl-tRNA-Synthetase kategorisiert. Das wichtigste Protein der zweiten Gruppe wurde als Katalase klassifiziert. Die Reduktion der Katalaseaktivität führt zu einer Reduktion der Wasserstoffperoxidkonzentration (H_2O_2). Proteine, die in den Energiestoffwechsel involviert sind, wie die Citratsynthetase wurden ebenfalls heruntergeregelt. Die Reduktion des Citratzyklusses ist eine normale Reaktion auf Stress (z. B. Salzstress). Die betroffenen ABC-Transporter, Oxydoreduktasen, O-succinylhomoserine sulfurhydrylase und 3-Hydroxy-butyrate dehydrogenase sind an sehr unterschiedlichen Stoffwechsel- und Biosynthesebereichen beteiligt. Dies lässt den Schluss zu, dass es sich bei den an der Salztoleranz beteiligten Genen nicht um ein einzelnes Regulon handelt.

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Appendix

Table A1. Standard mix of REP-PCR (25 µl).

Volume (µl)	Solution
10.4	H_2O (bid)
2.5	PCR buffer
5.2	$MgCl_2$ solution (25 mM)
2.5	DMSO (99.9%)
2.5	DNTP 's (12.5 mM for each)
0.3	R primer
0.3	F primer
0.3	*Taq* polymerase (10 U/µl)
1	DNA template

Table A2. Standard PCR mix (50 µl).

Volume (µl)	Solution
37.2	H_2O (bid.)
5	PCR buffer with 15 mM $MgCl_2$
2.5	DMSO
1	DNTP 's (10 mM for each)
1.5	R primer (10 pmol/µl)
1.5	F primer (10 pmol/µl)
0.3	Taq polymerase (10 U/µl)
1	DNA template

Table A3. Standard DIG-labelling PCR mix (50 µl).

Volume (µl)	Solution
33.2	H_2O (bid.)
5	PCR buffer with 15 mM $MgCl_2$
2.5	DMSO
5	10x DNTP labelling mix (10 mM for each)
1.5	sense primer (10 pmol/µl)
1.5	antisense primer (10 pmol/µl)
0.3	*Taq* polymerase (10 U/µl)
50 ng	DNA

Table A4. Rough estimated sizes of all 16S rDNA restriction fragments detected with the enzymes *HhaI*, *DdeI*, *MspI* and *Sau*3*AI*.

***HhaI* pattern**	**Representative strain**	**Estimated fragment size (bp)**	**Sum of fragments**
A	EBRI2,3, 21, 26 ,27, 32, *R. gallicum* R602sp, *R. giardinii* H152 and *R. etli* CFN 42	350,290,175,140,120,85	1160
B	EBRI 6, 20, 23 and 25 *Agrobacterium t.* 30150	370,340,290,175,140,120	1435
C	EBRI 24 and 29	370, 300, 220,175,140,120	1325
D	*R. tropici* CIAT 899	350,290,175,120,85	1020

***DdeI* pattern**	**Representative strain**	**Estimated fragment size (bp)**	**Sum of fragments**
A	EBRI 2,3,21,24,26,27, 29, 32, R. tropici CIAT 899, *R. gallicum* R602sp and *R. etli* CFN 42	430,380,280,260,150	1500
B	EBRI 6,20,23, 25, *R. giardinii* H152 and *Agrobacterium t.* 30150	380,350,280,260,150	1420

***MspI* pattern**	**Representative strain**	**Estimated fragment size (bp)**	**Sum of fragments**
A	EBRI 2,3,21, 26, 27, 32, *R. gallicum* R602sp and *R. etli* CFN 42	500,420,230,170,130	1450
B	EBRI 6, 20,23, 25 and *Agrobacterium t.* 30150	650, 270,250,180,65	1415
C	EBRI 24 and 29	650,500,180,150	1480
D	R. tropici CIAT 899 and *R. giardinii* H152	650,420,230,170	1470

***Sau*3*AI* pattern**	**Representative strain**	**Estimated fragment size (bp)**	**Sum of fragments**
A	EBRI 2,3,21,26, 27 and *R. etli* CFN 42	750,190	940
B	EBRI 6,20,23,25 and *Agrobacterium t.* 30150	1050,240,190	1480
C	EBRI 24 and 29	750,320,190,	1260
D	*R. tropici* CIAT 899	750,250, 190	1190
E	EBRI 32 and *R. gallicum* R602sp	950-190	1140
F	*R. giardinii* H152	380,360,250,200	1190

Table A5. Rough estimated sizes of the 23S rDNA restriction fragments detected with the enzymes *HinfI*, *MspI* and *Sau3AI*.

***HinfI* pattern**	**Representative strain**	**Estimated fragment size (bp)**	**Sum of fragments**
A	EBRI 2,3, 21,24, 26 ,27 and *R. tropici* CIAT899,	600,500,300,280,180,140,	2000
B	EBRI 6,20,23, 25 and *Agrobacterium t.* 30150	600,500,480,280,180	2040
C	EBRI 29	600,500,380,300,180	1960
D	*R. gallicum* R602sp and EBRI 32	880-480-280-260-180	2080
E	*R. etli* CFN 42	750-480-280-260-180	1950
F	*R. giardini* H152	480-460-280-260-180	1660

***MspI* pattern**	**Representative strain**	**Estimated fragment size (bp)**	**Sum of fragments**
A	EBRI 2,3,21,24, 26 and 27	580,420,380,260,240,85,65	2030
B	EBRI 6, 20,23, 25 and *Agrobacterium t.* 30150	750,500,380,240,85,65	2020
C	EBRI 29	800,450,380,320, 65	2015
D	*R. tropici* CIAT 899	630,420,380,250,160,110,85	2035
E	*R. gallicum* R602sp, *R. etli* CFN 42 and EBRI 32	Non digested fragment-430-380-260	1070
F	*R. giardini* H152	Non digested fragment-840-380-250	1470

***Sau3AI* pattern**	**Representative strain**	**Estimated fragment size (bp)**	**Sum of fragments**
A	EBRI 2,3,21,24,26 and 27	1490,290,260	2040
B	EBRI 6,20,23, 25 and *Agrobacterium t.* 30150	650,420,280,260,180,85	1875
C	EBRI 29	1480,410,180	2070
D	*R. tropici* CIAT 899	1480,290,240	2010
E	*R. gallicum* R602sp and EBRI 32	650-430-260-240-180-160-65	1985
F	*R. etli* CFN 42	1300-280-260-180-160	2180
G	*R. giardinii* H152	1350-280-260-165	2055

Table A6. Plasmid DNA content of rhizobial isolates and standard strains used in this study.

Strain	Number of plasmids	Size of plasmid kb
R. etli CFN42	6	650-510-390-270-180-150
EBRI2	6	650-510-400-390-260-65
EBRI3	4	650-400-390-180
EBRI29		
EBRI6	3	250-140-108
EBRI20	3	150-65-45
EBRI21	3	650-490-250
EBRI23	4	270-115-65-45
EBRI24	5	650-510-390-250-190
EBRI27		
EBRI 25	3	380-250-40
EBRI 26	5	650-510-390-250-208
EBRI32	2	550-250
R. tropici CIAT899		
R. gallicum Rsp602		
R. giardinii H152	2	140-120

Table A7. Copy number of *nifH* gene and their sizes of rhizobial isolates nodulating *Phaseolus vulgaris* from Egypt.

Strain	**Copy Nos of NifH gene**	**Size of NifH gene (kb)**	**Reference**
R. etli strains	3	9.8, 5.6, 4	Silva et al. (2003)
CFN 42			
EBRI 2	3	9.8, 5.6, 3.8	This study
EBRI 3	3	8.8, 5.6, 4.5	This study
EBRI 21	3	8.8, 5.6, 4.5	This study
EBRI 24	3	9.8, 5.6, 3.8	This study
EBRI 26	3	9.8, 5.6, 3.8	This study
EBRI 27	3	9.8, 5.6, 3.8	This study
EBRI 29	3	9.8, 5.6, 3.8	This study
R. gallicum strains	1	8.3	Sliva et al. (2003)
R602sp			
EBRI 32	1	7.2	This study
R. giardinii H152	0	0	Amarger et al. (1997)
R. tropici CIAT 899	1	-	Segovia et al. (1991)
Agrobacterium-like isolates			
EBRI 6, EBRI 20, EBRI 23 and EBRI 25	N.d	N.d	This study

N.d.: not determined.

Table A8. Characteristics of the proteins, over-expressed (14 spots) under salt stress in two replicates.

Replicate 1				Replicate 2			
Spot No.	**Vol ratio**	**Peak area**	**Peak height**	**Spot No.**	**Vol ratio**	**Area**	**Peak height**
944	15.1	858	2048	955	14.0	997	2068
1133	15.2	614	4269	1017	14.5	672	688
1629	15.9	1131	576	1675	14.6	1026	555
1162	16.7	1147	1734	1647	15.0	1188	465
247	18.5	736	1081	1629	15.2	1062	557
1034	18.9	626	670	1172	15.3	820	1386
880	20.8	526	638	259	17.1	1058	861
1668	23.0	875	489	1150	17.5	605	934
831	23.8	491	991	816	18.2	970	4631
796	27.2	735	5886	1042	19.4	630	684
1132	30.1	664	915	896	20.3	673	678
740	32.1	1259	9337	1155	21.1	1336	7399
766	49.3	442	7978	770	26.7	1822	8543
232	55.7	422	1826	241	40.2	527	1589

16S rDNA sequences of EBRI 2

ATCATGCTCAGAACGAACGCTGGCGGCAGGCTTAACACATGCAAGTCGAGCGCCCCGCAAGGG
GAGCGGCTACGGGTGAGTAACGCGTGGGAACGTACCCTTTACTACGGAATAACGCAGGGAAAC
TTGTGCTAATACCGTATGTGCCCTTCGGGGGAAGATTTATCGGTAAAGGATCGGCCCGCGTTG
GATTAGCTATTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCATAGCTGGKCTGAGAGGAT
GATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTGGGGAATAT
TGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGAGTGATGAAGGCCCTAGGGTTGTA
AAGCTCTTTCACCGGAGAAGATAATGACGGTATCCGGAGAAGAAGCCCCGGCTAACTTCGTGC
CAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGTTCGGAATTACTGGGCGTAAAGCGCACG
TAGGCGGATCGATCAGTCAGGGGTGAAATCCCAGGGCTAACCCTGGAACTGCCTTTGATACTG
TCGATCTGGAGTATGGAAGAGGTGAGTGGAATTCCGAGTGTAGAGGTGAAATTCGTAGATATT
CGGAGGAACACCAGTGGCGAAGGCGGCTCACTGGTCCATTACTGACGCTGAGGTGCGAAAGCG
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GGGCAGTATACTGTTCGGTGGCGCAGCTAACGCATTAAACATTCCGCCTGGGGAGTACGGTCG
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GGGTGC.

16S rDNA sequences of EBRI 3

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GGTGAGTAACGCGTGGGAACGTACCCTTTACTACGGAATAACGCAGGGAAACTTGTGCTAAT
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AGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCATAGCTGGTCTGAGAGGATGATCAGC
CACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACA
ATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGAGTGATGAAGGCCCTAGGGTTGTAAAGCT
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CGCAAGATTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTA
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GGGTTCCCTTCGGGGACCGGGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGA
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ACACACCGCCCGTCACACCATGGGAGTTGGTTTTACCCGAAGGTAGTGCGCTAACCGCAAGG
AGGCAGCTAACCACGGTAGGGTCAGCGACTGGGGTGCCGCCA.

16S rDNA sequences of EBRI 20

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TAAAACCAACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCAG

CATGCTGATCTGCGATTACTAGCGATTCCAACTTCATGCACTCGAGTTGCAGAGTGCAATCCG
AACTGAGATGGCTTTTGGAGATTAGCTCGACATCGCTGTCTCGCTGCCCACTGTCACCACCAT
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GAGCTGGTAAGGTTCTGCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCC
CCCGTCAATTCCTTTGAGTTTTAATCTTGCGACCGTACTCCCCAGGCGGAATGTTTAATGCGT
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CCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTCAGCGTCAGTAATGGACCAGT
AAGCCGCCTTCGCCACTGGTGTTCCTCCGAATATCTACGAATTTCACCTCTACACTCGGAATT
CCACTTACCTCTTCCATACTCAAGATACCCAGTATCAAAGGCAGTTCCAGAGTTGAGCTCTGG
GATTTCACCCCTGACTTAAATATCCGCCTACGTGCGCTTTACGCCCAGTAATTCCGAACAACG
CTAGCCCCCTTCGTATTACCGCGGCTGCTGGCACGAAGTTAGCCGGGGCTTCTTCTCCGGATA
CCGTCATTATCTTCTCCGGTGAAAGAGCTTTACAACCCTAAGGCCTTCATCACTCACGCGGCA
TGGCTGGATCAGGCTTGCGCCCATTGTCCAATATTCCCCACTGCTGCCTCCCGTAGGAGTTTG
GGCCGTGTCTCAGTCCCAATGTGGCTGATCATCCTCTCAGACCAGCTATGGATCGTCGCCTTG
GTAGGCCTTTACCCCACCAACTAGCTAATCCAACGCGGGCCAATCCTTCCCCGATAAATCTTT
CCCCCGTAGGGCGTATGCGGTATTAATTCCAGTTTCCCGGAGCTATTCCGCAGGAAAGGGTAT
GTTCCCACGCGTTACTCACCCGTCTGCCACTCCCCTTGCGGGGCGTTCGACTTGCATGTGTTA
AGCCTGCCGCCAGCGTTCGT.

16S rDNA sequences of EBRI 21

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GGGAGCGGCAACGGGTGAGTAACGCGTGGGAACGTACCCTTTACTACGGAATAACGCAGGGAA
ACTTGTGCTAATACCGTATGTGCCCTTCGGGGGAAAGATTTATCGGTAAAGGATCGGCCCGCG
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ACGTAGGCGGATCGATCAGTCAGGGGTGAAATCCCAGGGCTCAACCCTGGAACTGCCTTTGAT
ACTGTCGATCTGGAGTATGGAAGAGGTGAGTGGAATTCCGAGTGTAGAGGTGAAATTCGTAGA
TATTCGGAGGAACACCAGTGGCGAAGGCGGCTCACTGGTCCATTACTGACGCTGAGGTGCGAA
AGCGTGGGGAGCAAACAGGATTAGATCCCTGGTAGTCCACGCCGTAAaCGATGAATGTTAGCC
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16S rDNA sequences of EBRI 25

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TACACACCGCCCGTCACACCATGGGAGTTGGTTTTACCCGAAGGTAGTGCGCTAACCGCAAGG
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16S rDNA sequences of EBRI 26

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16S rDNA sequences of EBRI 27

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16S rDNA sequences of EBRI 32

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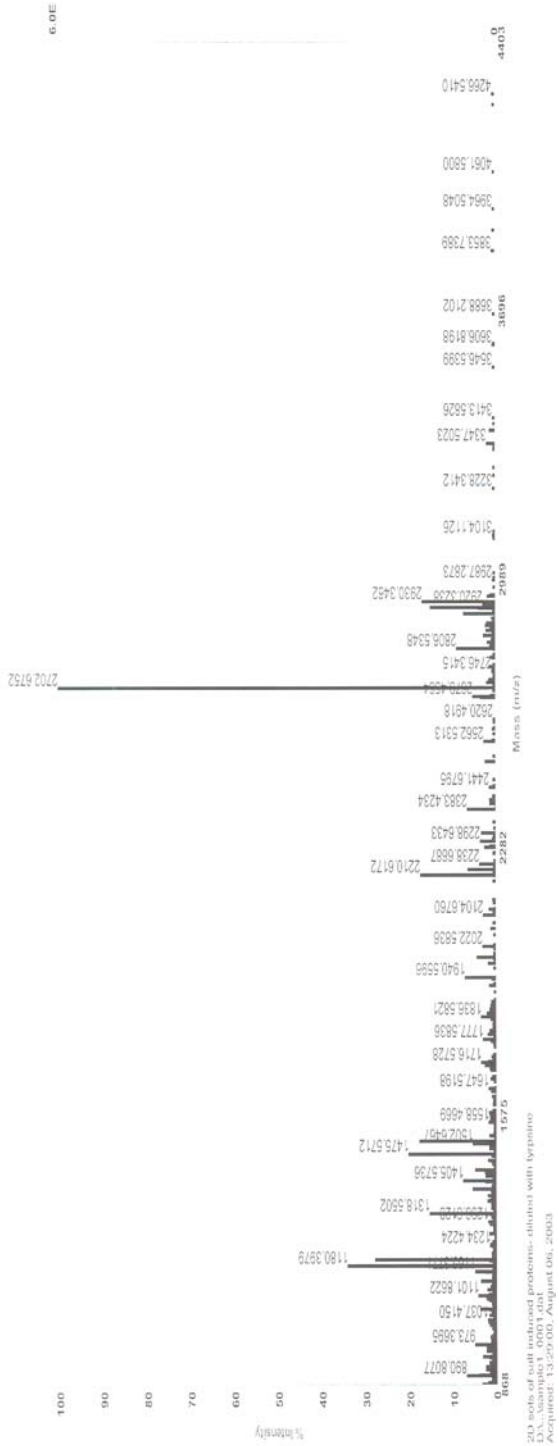
CAGAAGGTAGTGCCTAACCGCAAGGAGGCAGCT.

Figure A1. Nucleotide sequences of 16S rDNA for the Egyptian *Rhizobium* isolates, sequences are deposited at the Gene Bank under accession numbers from AY 221174 to AY 221181, corresponding to isolates EBRI 2, EBRI 3, EBRI 21, EBRI 26, EBRI 32, EBRI 27, EBRI 20 and EBRI 25 respectively.


6.0E
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2282
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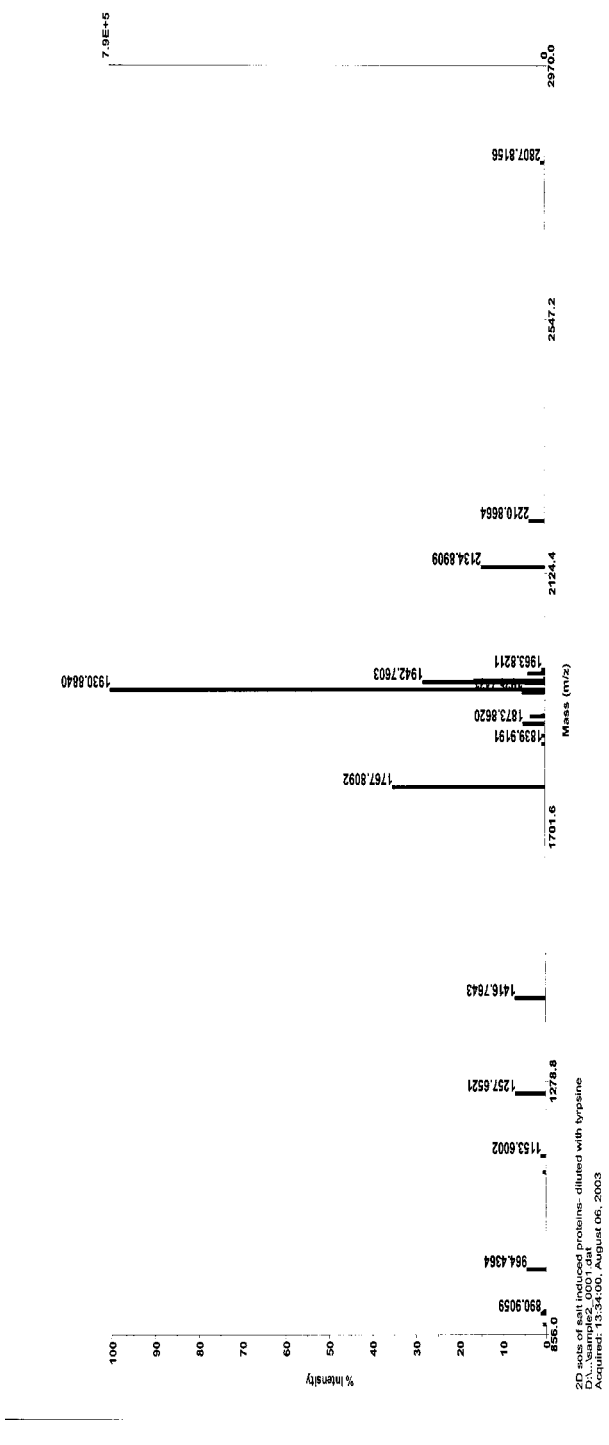
1037.4150
973.3865
890.8077
858
100
90
80
70
60
50
40
30
20
10
0
% Intensity
2D sols of salt induced proteins- diluted with tyrpsine
D:\...\Sample1_0001.dat
Acquired: 13:29:00, August 06, 2003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0
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2547.2
2210.8664
2134.8909
2124.4
1963.8211
1942.7603
1930.8440
1873.8620
1839.9191
1767.8092
1701.6
Mass (m/z)
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1278.8
1257.6521
1153.6002
964.4364
890.9059
856.0
100
90
80
70
60
50
40
30
20
10
0
% Intensity
2D sols of salt induced proteins- diluted with tyrpsine
D:\...\Sample2_0001.dat
Acquired: 13:34:00, August 06, 2003

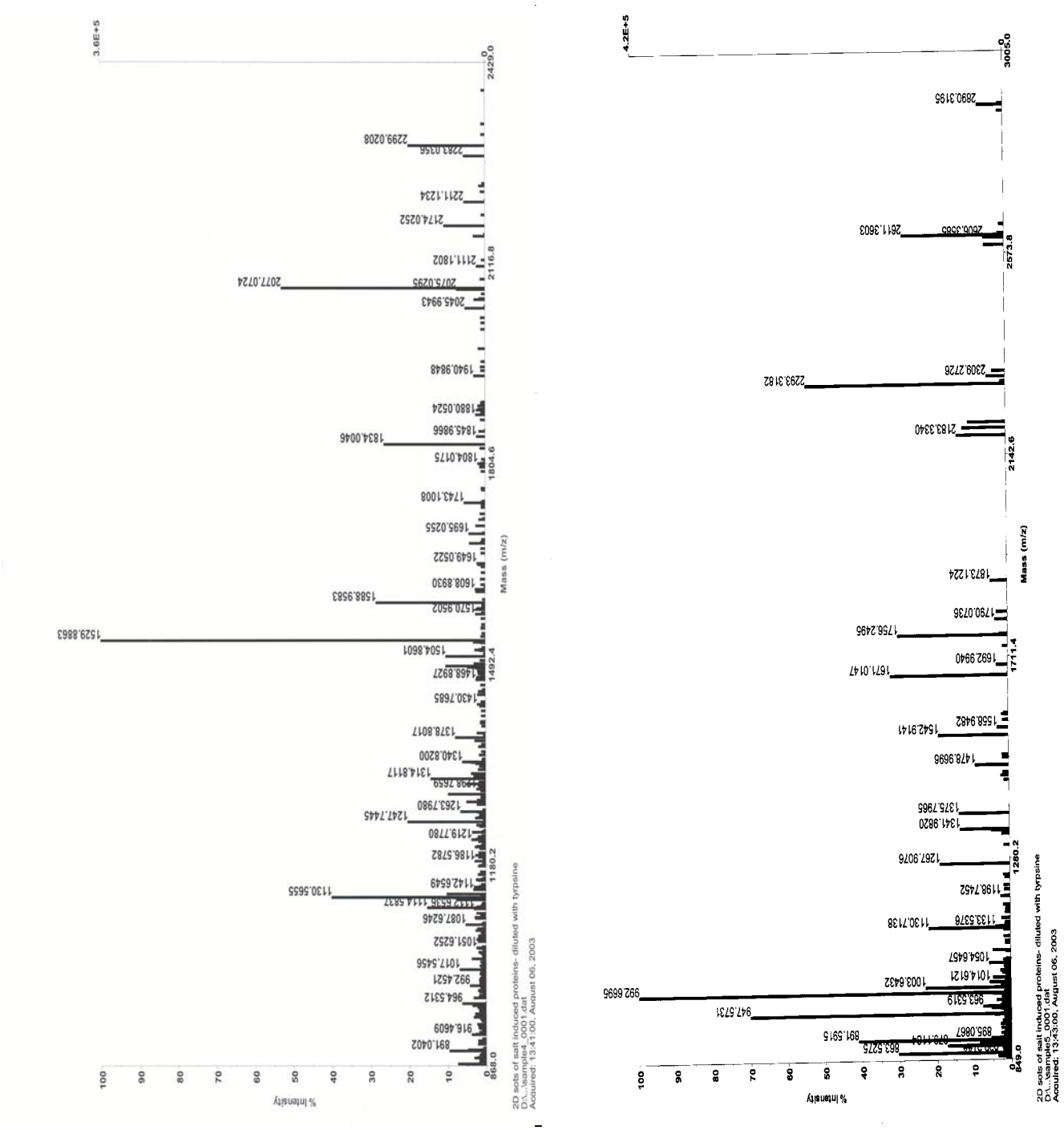

Figure A2. Mass finger print of the interested spots of proteins from *R. etli*, digested with tyrpsin and analysed by MALD-TOF machine.

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Erklärung

Ich versichere, dass ich meine Dissertation
„Improvement of common bean (*Phaseolus vulgaris*) nodulation by selected rhizobial strains from Egyptian soils through genotypic characterization, symbiotic effectiveness and competitiveness under salt stress conditions“
selbständig, ohne unerlaubte Hilfe angefertigt und mich dabei keiner anderen als der von mir ausdrücklich bezeichneten Quellen und Hilfen bedient habe.

Die Dissertation wurde in der jetzigen oder einer ähnlichen Form noch bei keiner anderen Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.

Marburg, den ……

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Selection of competitive strains of *Rhizobium* nodulating *Phaseolus vulgaris* and adapted to environmental conditions in Egypt, using the *gus*-reporter gene technique

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Summary

Two *Rhizobium etli* strains, EBRI 2 and EBRI 26, isolated from Egypt were tested for nodulation competitiveness on beans using *Rhizobium tropici* CIAT 899G as the competing strain. The insertion of the *gus*-reporter transposon mTn5ssgusA30 did not alter the nodulation or nitrogen fixation capacity of mutant strain CIAT 899G compared to the wild type. At neutral pH, *R. etli* strains EBRI 2 and EBRI 26 were more competitive than CIAT 899G with the bean cultivar Saxa. These two strains gave nodule occupancies of 52.1 and 61.1% competing with equal cell numbers of CIAT 899G. Nodule occupancies from these two native strains increased with the bean cultivar Giza 6 from Egypt to 66 and 67.5%. Based on these results, cultivar Giza 6 was used to select the most competitive strains under stress of salinity or alkalinity as a major problem for a large part of Egyptian soils. Under stress of salinity (0.2% NaCl or 34.2 mM NaCl), the salt-sensitive strain EBRI 2 was more competitive than the salt-resistant strain EBRI 26. Strain EBRI 2 gave 87.4% but strain EBRI 26 gave 63.7% nodule occupancy against CIAT 899G. The same trend of results was observed under stress of alkalinity (pH 8). Strain EBRI 2 occupied 83% while Strain EBRI 26 occupied 53.2%.

Introduction

Competitiveness in rhizobia is the ability of a given strain to infect a legume host and form nodules in the presence of other strains. Strains that dominate nodules are considered more competitive than other strains. The success of inoculation requires, that the inoculum strain must be both highly effective in nitrogen fixation and highly competitive against the native strains in the soil (Williams & Phillips 1983; Singleton & Tavares 1986; Segovia *et al.* 1991). To evaluate competitiveness, it is necessary to identify a specific strain of rhizobia within nodules. Several different techniques have been used to determine nodule occupancy including fluorescent antibodies (Schmidt *et al.* 1968), antibiotic resistance (Turco *et al.* 1986), plasmid profiles (Shishido & Pepper, 1990) and specific gene probes (Judd *et al.* 1993; Richardson *et al.* 1995). DNA markers such as the *gus* gene are especially useful techniques to mark rhizobial strain for studying the competitiveness. The *gus* operon codes for the β-glucuronidase enzyme and it is used as a DNA marker for detecting of bacteria in the rhizosphere or in association with plants (Wilson *et al.* 1991). Bean nodules infected by rhizobial strains with the *gus* gene stain blue when incubated with x-gluc buffer (Wilson 1995). Many investigators (Streit *et al.* 1992, 1995; Sessitsch *et al.* 1997, 1998; Anyango *et al.* 1998; Diouf *et al.* 2000) have used this technique to identify rhizobial strains inside nodules after inoculation with competing strains. The majority of soils contain high numbers of indigenous rhizobial strains which are often ineffective in symbiosis but highly competitive due to their adaptation to given environmental conditions. The persistence of these indigenous strains often limits the success of inoculation, consequently it is important to select the most effective and competitive strains from new isolates (Sessitsch *et al.* 1998). Therefore, we tested the ability of *R. etli* strains EBRI 2 or EBRI 26 from Egyptian soils in competition against the standard strain *R. tropici* CIAT 899G in order to evaluate their competitive rank.

Materials and methods

Bacterial strains

Two strains of *R. etli* EBRI 2 and EBRI 26, salt-sensitive and salt-resistant, respectively from Egyptian

soils, were used in this study together with *R. tropici* CIAT 899. *E. coli* strain S17-1-λ-pir carrying the plasmid PCAM130 (Wilson *et al.* 1995) was used as a donor strain. *R. etli* and *R. tropici* strains were cultivated in 20E medium (Werner *et al.* 1975) at 28 °C for 48 h. The *E. coli* strain was cultivated in LB medium (Sambrook *et al.* 1989), containing spectinomycin (50 μg ml^{-1}) at 37 °C until the mid exponential phase.

Transposon mTn5ssgusA30

Wilson *et al.* (1995) designed *gus*A transposon mTn5ssgusA30 which contains a nifH promoter upstream of *gus*A.

Construction of a R. tropici CIAT 899 gus$^+$ strain

R. tropici CIAT 899 integrated the *gus* gene from the mobilizable plasmid pCAM130, from donor strain S17-1-λ-pir (de Lorenzo *et al.* 1990) by conjugation (Simon *et al.* 1983). Three loopfuls of *E. coli* and three loopfuls of *R. tropici* were mixed and left for patch mating on PY plates (Noel *et al.* 1984), by incubation at 28 °C overnight. Bacteria were transferred into 5 ml of 0.85 NaCl plus 0.05 Tween 20 to remove an excess of exopolysaccharides, and diluted to 10^{-4}. Hundred microlitre of the dilution 10^{-4} were plated on MM medium (Kingsly & Bohlool 1992) amended with 50 μg spectinomycin and streptomycin ml^{-1} to select the recipient strain of *R. tropici* with Tn5ssgusA30. Colonies were re-streaked on the MM plus 50 μg spectinomycin and x-gluc substrate ml^{-1} to screen for spectinomycin-resistant colonies marked with the *gus* gene. Blue colonies were recognized, picked and sub-cultured on the same medium to check the purity and the *gus* stability of the colonies. Colonies marked with *gus* gene were kept with 50% glycerol at −70 °C for competition studies.

Gus gene activity assays

Nodulated roots of common bean were soaked in β-glucosidase staining buffer with 4.35 g K_2HPO_4, 3.41 g KH_2PO_4, 5 ml of SDS (10%), 2.8 ml of sarcosyl (35%), 1 ml of Triton X-100, 0.37 g EDTA, 50 mg of x-gluc (dissolved in DMF), H_2O up to one litter and pH between 7.5 and 8. The whole root systems were incubated in the dark on a rotary shaker at 28 °C for 48 h.

Plant nodulation assays and competition experiments

The nodulation assays were performed in sterilized Leonard jars with vermiculite: perlite (1:1 v:v) as a medium for cultivation and N-free nutrient solution (Werner *et al.* 1975). Seeds of common bean cultivars Saxa and Giza 6 from Germany and Egypt, respectively, were surface sterilized (Vinuesa *et al.* 1998) and distributed on the surface of 1% agar plates, then incubated for 3–4 days at 28 °C for germination. After germination, seedlings were transferred to Leonard jars for competition experiments. Seeds were inoculated by adding 1 ml of rhizobial cultures at the mid of exponential growth phase with 10^8 cells ml^{-1} for each Leonard jar. Strains were mixed in a 1:1 ratio for each treatment. Plants were cultivated in a controlled growth chamber with 15 h of light at 25 °C and 9 h for darkness at 18 °C, relative humidity of 75% and light intensity of 14 k Lux. To study the competition under stress of salinity, sodium chloride was added to the nutrient solution at a final concentration 0.2% NaCl (34.2 mM). For alkaline stress, the nutrient solution was buffered with 20 mM AMPD for a pH8 (Priefer *et al.* 2001). Each treatment was replicated three times. Plants were harvested 25 or 30 days after inoculation. Acetylene reduction assays (ARA) was estimated as described (Bender & Rolfe 1985) and nodulation parameters were recorded. Part of the nodulated roots were used for the *gus* reporter gene assay. Competitiveness indices were estimated using the equation described by Beattie *et al.* (1989).

Results and discussion

Competition between CIAT 899 and the gus fusion derivative CIAT 899G

The first step in our experiments was the construction of a *gus* reporter gene strain. Strain CIAT 899G with the *gus* gene produced blue colonies compared with the wild type when grown on MM amended with 50 μg x-gluc ml^{-1} as observed in the previous reports. No statistically significant differences were detected in nodule number among plants inoculated with CIAT 899G or the parent strain CIAT 899 (Table 1). This revealed the suitability of CIAT 899G for a quantitative analysis of nodule occupancy in co-inoculation experiments, after proof that this strain is stable and not significantly affected in its competitive ability due to the insertion of reporter gene. This has also been shown in previous reports (Streit *et al.* 1995; Sessitsch *et al.* 1997).

Effect of host cultivar on nodulation and competition under neutral pH

The number of nodules developed by inoculation with Egyptian strains EBRI 2 and EBRI 26 were not affected by co-inoculation with strain CIAT 899G (Table 1). This result was obtained with the cultivar Giza 6 from Egypt as well as with the cultivar Saxa from Europe. It is remarkable that the root weight of both cultivars is almost the same in the non-inoculated control plants as in those plants, which produce after symbiotic infection a large amount of nodules. The weight of the nodules exceeds the weight of the roots by a factor of two or more. However, this ratio was significantly lower, when a co-inoculation of the strain EBRI 2 or EBRI 26 with the CIAT 899G was used. This effect was only observed

Table 1. Nodulation and nitrogen fixation of single or dual strain inoclum with two host cultivars, 30 days after inoculation (pH 6.85).

Treatments	No. of nodules plant^{-1}	Fresh weight of nodules mg plant^{-1}	Fresh weight of shoot g plant^{-1}	Fresh weight of root mg plant^{-1}	nmol C_2H_4 h^{-1} mg^{-1} nodules (ARA activity)
Bean cultivar Giza 6					
Control	0.0b	0.0d	4.6d	440	0.0c
Inoculated with CIAT 899Wt	229a	1103b	7.9b	390	13.7ab
Inoculated with CIAT 899G	236a	1648a	13.2a	520	6.9b
Inoculated with EBRI 2	231a	1055b	10.3b	450	13.5ab
Inoculated with EBRI 26	214a	963b	10.2b	450	11.6b
Inoculated with CIAT 899G + EBRI 2	200a	675cb	7.5cb	470	16.2ab
Inoculated with CIAT 899G + EBRI 26	139a	610cb	8.1bc	550	15.3ab
Bean cultivar saxa					
Control	0.0b	0.0d	2.7ed	450	0.0c
Inoculated with CIAT 899 Wt	178a	795bc	4.9d	380	11.3b
Inoculated with CIAT 899G	147a	563cb	5.4cd	420	14.8ab
Inoculated with EBRI 2	159a	616cb	5.1d	360	15.5ab
Inoculated with EBRI 26	169a	605cb	5.5cd	410	17.5a
Inoculated with CIAT 899G + EBRI 2	221a	748bc	6.1cd	480	11.7ab
Inoculated with CIAT 899G + EBRI 26	173a	512cb	5.1d	460	15.9a
LSD 0.05	NS	391.69	2.27	NS	5.18

Wt wild type, CIAT 899G marked with *gus*-gene, EBRI Egyptian bean rhizobial isolates, NS not significant, letters on the right side indicating on the statistical analysis.

with the Egyptian cultivar Giza 6. The specific ARA activity per plant was rather similar in all variations. The higher nodule weight in the plants inoculated with CIAT 899G produced a lower specific activity per mg nodule weight. To estimate the nodule occupancy, roots with dual strain inoculation were assayed for β-glucosidase by incubation in *gus* staining buffer to recognize the blue and white nodules. The staining of nodules was strong due to the plasmid mTn5ssgusA30 used, containing the nifH promoter of *R. etli* strain CFN42 and an upstream activating sequence responsible for an enhanced activity of *gus* gene in nodules (Wilson *et al.* 1995). Results of competition between the Egyptian strains *R. etli* EBRI 2 or EBRI 26 with the strain CIAT 899G under neutral pH are presented in Table 2. Strain EBRI 2 is a strong competitor against strain CIAT 899G only in cultivar Giza 6 (66% nodule occupancy), strain EBRI 26 was also very competitive with 67.5% nodule occupancy. The results are in agreement with those obtained by George & Robert (1992) who mentioned that *R. tropici* CIAT 899 was moderately competitive. Anyango *et al.* (1998) found that *R. tropici* CIAT 899 had only 14% of nodule occupancy when examined against *R. etli* Kim5 in a near-neutral pH soil, but formed 35% of the nodules at acid pH. A small percent of nodules (0.61–1.2%) were partially staining blue, indicating double nodule occupancy, as observed by Krishnan & Pueppke (1992). Competitiveness indices were estimated using the equation described by Beattie *et al.* (1989). The results indicate that the Egyptian rhizobial strains are highly competitive against CIAT 899G.

Table 2. Nodule occupancy of *R. etli* EBRI 2 and EBRI 26 at neutral pH, 30 days from inoculation in competition with *R. tropici* CIAT 899G marked with *gus* gene.

Strains used	% Nodule occupancy by EBRI 2	% Nodule occupancy by CIAT 899G	% Dual nodule occupancy	Competitiveness index C X:Y	Host cultivar
CIAT 899G + EBRI 2	52.1 ± 3.5	46.8 ± 4.5	1.1 ± 0.6	0.05	Saxa
CIAT 899G + EBRI 2	66 ± 3.2	33.39 ± 1.8	0.61 ± 0.6	0.29	Giza 6
	% Nodule occupancy by EBRI 26	% Nodule occupancy by CIAT 899G	% Dual nodule occupancy	Competitiveness index C X:Y	Host cultivar
CIAT 899G + EBRI 26	61.1 ± 3.57	38.9 ± 2.6	0.00	0.19	Saxa
CIAT 899G + EBRI 26	67.5 ± 2.9	31.3 ± 2.8	1.2 ± 0.53	0.33	Giza 6

X: EBRI strain, Y: CIAT 899G, results are means of three replicates ± standard errors.

Table 3. Nodulation and nitrogen fixation of bean cultivar Giza 6 under stress of salinity (34.2 mM NaCl), after 25 days of inoculation.

Treatments	No. of nodules plant^{-1}	Fresh weight of nodules mg plant^{-1}	Fresh weight of shoot g plant^{-1}	Fresh weight of root mg plant^{-1}	nmol C_2H_4 h^{-1} mg^{-1} nodules (ARA activity)
Control	0.0b	0.0c	3.4c	350	0.0c
Inoculated with CIAT 899Wt	69a	390ba	5.6ba	470	12.4a
Inoculated with CIAT 899G	97a	641a	7.6a	430	10.3a
Inoculated with EBRI 2	144a	706a	7.7a	430	8.9ba
Inoculated with EBRI 26	98a	568a	6.9a	420	10.2a
Inoculated with EBRI 2 + 899G	82a	460a	6.8a	420	10.8a
Inoculated with EBRI 26 + 899G	83a	343ba	7.4a	360	13.3a
LSD 0.05	NS	284.69	1.52	NS	3.55

Wt wild type, CIAT 899G marked with *gus* gene, NS non significant, letters on the right side indicating on the statistical analysis.

Competition under stress of salinity

Results in Table 3 present the nodulation parameters of single or dual strain inoculation under stress of salinity using the cultivar Giza 6. There were no significant differences among nodule numbers formed. The amount of nodule fresh weight never exceeded the root fresh weight by more than a factor of 2. The ARA activity per plant was again rather similar in all combinations. Generally, the stress of salinity decreased the nodule number, nodule fresh weigh and shoot fresh weight comparing with the growth of plants under normal conditions (Table 1). These results are in agreement with reports by Bekki *et al.* (1987) and by Bordeleau & Prevost (1994). The percentages of nodule occupancy under stress of salinity are listed in Table 4. It is interesting to note that the salinity-sensitive strain EBRI 2 (tolerance up to 0.5% NaCl) was a better competitor against CIAT 899G than the highly salt-resistant strain EBRI 26 (tolerant up to 4% NaCl). Strain EBRI 2 occupied 87.4% of the nodules while strain EBRI 26 occupied only 63.7% against CIAT 899G. These results are in agreement with those obtained from Subba Rao *et al.* (1990) who found, that the *Rhizobium* strains well adapted to form effective symbiosis with their hosts at saline conditions are not necessarily obtained from saline soils. Also, Nair *et al.* (1993) noticed that some rhizobial strains tolerating extremely high levels of salt, had a significantly lower symbiotic efficiency under salt stress. This indicates that competitiveness of strains under stress is not only due to the ability of strains to resist the increasing level of sodium chloride but also to other factors such as motility and chemotaxis (Mellor *et al.* 1987; Zdor & Pueppke 1991), cell surface polysaccharides (Handelsman *et al.* 1984; Lagares *et al.* 1992) and bacteriocin production (Triplett & Barta 1987). Strain EBRI 2 has a high rate of exopolysaccharide production.

Competition under stress of alkalinity

Results in Table 5 give nodulation parameters of single or mixed strain inoculation under stress of alkalinity. In all cases the nodule numbers were higher than under salt stress (Table 3). The percentages of nodule occupancy are listed in Table 4. Although the strains EBRI 26 and

Table 4. Nodule occupancy of *R. etli* EBRI 2 and EBRI 26 under stress of salinity and alkalinity with bean cultivar Giza 6, 25 or 30 days after inoculation competing with *R. tropici* CIAT 899G marked with *gus* gene.

Strains used	% Nodule occupancy by EBRI 2 or EBRI 26	% Nodule occupancy by CIAT 899G	% Dual nodule occupancy	Competitiveness index C X:Y	Stress of salinity
CIAT 899G + EBRI 2	87.4 ± 2.5	9.4 ± 1.8	3.2 ± 0.76	0.86	34.2 mM NaCl
CIAT 899G + EBRI 26	63.7 ± 5.2	33.1 ± 4.3	3.2 ± 1.3	0.27	34.2 mM NaCl
	Nodule occupancy by EBRI 2 or EBRI 26	Nodule occupancy by CIAT 899G	Dual nodule occupancy	Competitiveness index	Stress of alkalinity C X:Y
CIAT 899G + EBRI 2	83 ± 4.4	12.4 ± 2.5	4.6 ± 1.7	0.71	pH 8
CIAT 899G + EBRI 26	53.2 ± 5.8	43.9 ± 5.8	2.9 ± 1.4	0.08	pH 8

X: EBRI strain, Y: CIAT 899G, results are means of three replicates ± standard errors.

Table 5. Nodulation and nitrogen fixation of bean cultivar Giza 6 under stress of alkalinity at pH 8, 30 days after inoculation.

Treatments	No. of nodules plant^{-1}	Fresh weight of nodules mg plant^{-1}	Fresh weight of shoot g plant^{-1}	Fresh weight of root mg plant^{-1}	nmol C_2H_4 h^{-1} mg^{-1} nodules (ARA activity)
Control	0.0c	0.0c	4.6b	510a	0.0c
Inoculated with CIAT 899Wt	158ab	1156a	9.7a	560a	14.2b
Inoculated with CIAT 899G	163ab	980a	9.5a	480ba	14.9b
Inoculated with EBRI 2	136ba	671ab	8.4ab	520a	19.8a
Inoculated with EBRI 26	127ba	657b	7.9ab	480ba	19.2a
Inoculated with EBRI 2 + 899G	159ab	930a	8.4ab	520a	14.3b
Inoculated with EBRI 26 + 899G	182a	675ba	6.5ba	430b	16.6ab
LSD 0.05	40.1	269.81	2.01	0.08	3.39

Wt wild type, CIAT 899G marked with *gus* gene, letters on the right side indicating on the statistical analysis.

CIAT 899G are more resistant to alkalinity in liquid culture (maximum level of growth pH 9) than strain EBRI 2 which was moderately tolerant to alkaline pH (maximum level of growth pH 8.5), strain EBRI 2 gave the highest percent of nodule occupancy 83% compared with EBRI 26 (53.2%) against CIAT 899G, respectively.

Conclusions

The *gus* reporter gene can be used to select the most competitive strains and also to determine double nodule occupancy. Strain *R. tropici* CIAT 899 is an effecive inoculum strain and its ability to fix atmospheric nitrogen is not affected as a result of *gus* insertion. The newly isolated *Rhizobium etli* strains EBRI 2 and EBRI 26 are more competitive than strain CIAT 899G. We can use these strains as inoculants for Egyptian fields, but we recommend the use of strain EBRI 2 in soils with high salt or alkaline conditions.

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Rhizobium etli and *Rhizobium gallicum* Nodulate *Phaseolus vulgaris* in Egyptian Soils and Display Cultivar-Dependent Symbiotic Efficiency

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Abstract

Twelve rhizobial isolates were recovered from nodules of common bean (*Phaseolus vulgaris*) grown in two different locations of Egyptian soils. The most effective strains for nodule formation and nitrogen fixation were selected. Strain specificity with the bean cultivars Saxa, Canoca and Giza 6 from Germany, Colombia and Egypt were studied. The strains were characterized by amplified rDNA restriction analysis of 16S and 23S rDNA (ARDRA), plasmid DNA content and 16S rDNA sequencing. A high degree of genetic diversity was observed among the strains used. The strains were separated into three genotype groups. Genotype A was displayed by seven isolates classified as *Rhizobium etli*, while genotype B was displayed by a single isolate, classified as *R. gallicum*. Genotype C included four isolates which were unable to re-nodulate *Phaseolus vulgaris*, which were

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related to *Agrobacterium tumefaciens*. Single strains were further characterized by specific physiological tests and measurements such as acetylene reduction activity, nodule/root biomass ratio, shoot and root fresh weight.

Keywords: *Phaseolus vulgaris*, Egypt, rhizobia, molecular characterisation

1. Introduction

Phaseolus vulgaris has its origin in Central and South America. It is a major world crop with almost 20 million tons of seeds produced per year, ranking directly after soybeans and peanuts in the world production of grain legumes (FAOSTAT, 2001). The area on which common beans are produced is more than 27 million hectares, with an average production is around 7 dry ton (dt) per hectar (ha). High production rates are found in Germany with 26 dt per ha whereas the production rate in African countries is around 7 dt per ha. This means, there is a large potential for increasing the production of *Phaseolus vulgaris* in Africa (Kratzsch, 1999).

In Egypt, common bean is widely consumed as vegetables and also as dry seeds. The annually cultivated area ranges between 9–18 thousand hectares, yielding 15,000 tons of dry seeds and 150,000 tons of green pods (data from the Central Administration of Agriculture in Egypt CAAE, 1994). Different *Rhizobium* species are able to nodulate and fix nitrogen with the common bean (*Phaseolus vulgaris*), including *Rhizobium leguminosarum* bv. *phaseoli* (Jordan, 1984), *R. tropici* (Martinez et al., 1991), *R. etli* (Segovia et al., 1993), *R. gallicum* and *R. giardini* (Amarger et al., 1997). The percentage of biological nitrogen fixation on the N assimilation in *Phaseolus vulgaris* is, compared to other legumes, rather low, with 40–50% compared to 75% with faba beans, 70% with peas and up to 95% with lupines (Werner, 1999).

Failure of nodulation or lack of response to inoculation in the field has been reported, raising doubts about the benefits of inoculation (Buttery et al., 1987, Graham, 1981). Factors that can limit inoculation benefits are the promiscuity observed in *Phaseolus vulgaris* (Hernandez et al., 1995, Michiels et al., 1998), environmental conditions (Robert and Schmidt, 1983), the effect of high N-fertilizer concentrations used in intensive agriculture (Temprano et al., 1997) and genomic rearrangements in the micro-symbionts (Girard et al., 1991). The genetic biodiversity of *Rhizobium* in bean nodules is significantly affected by soil fertilization, especially by ammonia and nitrate (Caballero-Mellado and Martinez-Romero, 1999).

There are reports from different regions in the world stressing both genetic uniformity (Wegener et al., 2001) and large biodiversity of bean nodule isolates (Castro-Sowinski et al., 2002) but little information is available for Egyptian

bean-nodulating rhizobia. Based on these reports our research had the aim of (i) isolating new effective bean nodule isolates from Egyptian soils, characterizing their nodulation efficiency on bean cultivars from Egypt and other countries, (ii) to study the genetic diversity of these strains by molecular methods such as ARDRA techniques (Vinuesa et al., 1998), plasmid profiling (Kuykendall and Hashem, 1994) and to classify them on the basis of 16S rDNA phylogeny (Amarger et al., 1997).

2. Materials and Methods

Isolates and reference strains

Twelve rhizobial isolates were obtained from the root nodules of common bean (*Phaseolus vulgaris*) grown in two different locations of Egyptian soils. Isolates EBRI 2, 3, 6, 25 and 29 were from the Isma'ilya desert while isolates EBRI 20, 21, 23, 24, 26, 27 and 32 were from the Ashmun area in the Nile Valley Delta. Plant samples were collected by digging the root system with a block of soil. The moist blocks were transported in polyethylene bags to the laboratory for isolation of rhizobia. Rhizobia were isolated from surface-sterilized nodules according to the method described by Somasegaran and Hoben (1985). *R. tropici* CIAT 899T, *R. etli* CFN 42T and *A. tumefaciens* DSM30150 were obtained from culture collection of the Laboratory of Cell Biology and Applied Botany in Marburg. Strains of *R. gallicum* R602spT and *R. giardini* H152T were obtained from N. Amarger. Isolates and standard strains were preserved in 50% glycerol at –70°C for further studies.

Phenotypic characteristics

Strains were examined for growth on LB medium and on a basal medium with D-glucuronate as a main carbon source or with 5 μg ml^{-1} spectinomycin as done by Amarger et al. (1997). Also, strains were tested for growth on minimal medium MM containing malate or sorbitol as a sole carbon source, as reported by Segovia et al. (1993).

Bean cultivars

Three cultivars of *Phaseolus vulgaris* were used in this study. The Saxa and Canoca cultivars were obtained from the germplasm collection of the Department of Biology, Philipps-University Marburg, Germany and the Giza 6 cultivar was obtained from the Institute of Vegetable Crops Research, Ministry of Agriculture, Egypt.

Plant nodulation assays

The nodulation assays were performed in sterilized growth pouches or in Leonard jars with vermiculite: perlite (1:1 v:v) as substrate and N-free solution (Werner et al., 1975). Seeds of common bean were surface sterilized according to Vinuesa et al. (1998) and distributed on the surface of 1% agar plates and incubated for 3–4 days at 28°C for germination. After germination seedlings were transferred to growth pouches for nodulation experiments or to Leonard jars for host specificity experiments under sterile conditions. Seeds of *Leucaena leucocephala* cv. Cunningham were scarified for 20 min in concentrated H_2SO_4, surface sterilized and cultivated as previously described for common bean seeds (Vinuesa et al., 1998). Seeds were inoculated by adding 1 ml of rhizobial cultures at the mid of the exponential growth phase with about 10^8 cells ml^{-1} for each Leonard jar. Seeds cultivated in growth pouches, were inoculated by soaking for three minutes in one ml of a 1:20 diluted inoculum suspension.

Plants were cultivated in a controlled growth chamber with 15 h of light at 25°C and 9 h for darkness at 18°C and relative humidity of 75%. Light intensity was 14 k Lux. Plants were harvested 21 or 30 days after inoculation. Acetylene reduction assays (ARA) were performed as described by Bender and Rolfe (1985).

Isolation of DNA

Genomic DNA of the strains was obtained from liquid culture at the mid exponential phase using cetyltrimethyl amoniumbromide (Ausubel et al., 1994). Purified DNA was dissolved in T.E. buffer (10 mM Tris-HCl and 1 mM EDTA pH. 8.0). The concentration of DNA was adjusted to 50 $\mu g\ ml^{-1}$.

Amplified Ribosomal DNA Restriction Analysis (ARDRA)

Primers fD1 and rD1 were used to amplify nearly full-length 16S rDNA genes (Weisburg et al., 1991), while the primers P3 and P4 were used to amplify the region of 23S rDNA described by Terefework et al. (1998). The PCR reaction was performed using the standard reaction mixture (50 μl) containing 1x PCR buffer, 1.5 mM $MgCl_2$, 5% dimethyl sulfoxide, 200 μM for each nucleotide (Boehringer GmbH, Mannheim, Germany), 15 pmol of each primer, 1 U of taq polymerase, and 50 ng of purified DNA. The temperature program was as follows: initial denaturation at 95°C for 3 min 30 s; 35 cycles of denaturation at 94°C for 70 s, anneling temperature at 56°C for 40 s and extension at 72°C for 130 s, and final extension at 72°C for 370 s (Vinuesa et al., 1998).

PCR products from 16S rDNA amplification experiments were digested with the four restriction enzymes *Hha1*, *Dde1*, *Msp1* and *Sau3a1*, while the amplified fragments of 23S rDNA amplicons were restricted with *Msp1*, *Sau3a1* and *Hinf1* (USB Amersham International), as recommended by the manufacturer.

The restriction patterns were resolved by electrophoresis in a 2% agarose gel in TBE buffer (Tris-Borate EDTA) at 55 v for 3 h, as described by (Vinuesa et al., 1998). The RFLP patterns were normalized using a 100 bp ladder from GIBCO BRL (Eggenstein, Germany). The gels were stained post-electrophoresis with ethidium bromide.

Analysis of rDNA restriction patterns

Gel images were digitised using a charge coupled device video camera (INTAS, Göttingen, Germany) and stored as TIFF files. Data were analysed using the Gel Compar II software package (version 2.0; Applied Maths, Kortrijk, Belgium) as described in detail elsewhere (Vinuesa et al., 1998).

Plasmid DNA content

Plasmid profile analysis for twelve rhizobial isolates EBRI 2, 3, 6, 20, 21, 23, 24, 25, 26, 27, 29 and 32 in addition to *R. tropici* (CIAT 899T), *R. gallicum* (R602spT) and *R. giardini* (H152T) and *R. etli* (CFN42T) was done according to the modified Eckhardt procedure of Kuykendall and Hashem (1994).

Direct sequencing of 16S rDNA

Amplified 16S rDNA fragments were purified using the Quiaquick high pure PCR purification kit (QIAGEN, Düsseldorf, Germany) according to manufacture's instructions. Primers fD1, rD1 and four internal primers (5' - CCG GGC GGT GTG TAC AGG G - 3'; 5' - TAC GGG AGG CAG CAG - 3'; 5' - GTC AAT TCC TTT GAG - 3' and 5' - CCG GGC GGT GTG TAC AAG G - 3') were used to obtain full-length readings of both strands of the *rrs* amplicon. Cycle-sequencing was performed using the AmpliTaq-DNA-polymerase, based on the dideoxy chain terminator technique of Sanger et al. (1977). DNA sequences were read on a 373A (Applied Biosystems). Sequence reads were edited and assembled using DNASTAR (Lasergene) software. The 16S rDNA sequence for 8 Egyptian isolates were deposited at GeneBank under the accession numbers from AY 221174 to AY 221181, corresponding to isolates EBRI 2, EBRI 3, EBRI 21, EBRI 26, EBRI 32, EBRI 27, EBRI 20 and EBRI 25, respectively.

16S rDNA sequence alignment and phylogeny estimation

Sequence similarity searches were performed at the NCBI server using BLASTN (http://www.ncbi.nlm.nih.gov/blast). Multiple nucleotide sequence alignments were generated and edited using ClustalW, as implemented in BioEdit (Hall, 1999). The *rrs* multiple sequence alignment was manually adjusted to fit that produced by the Ribosomal Database Project-II (Maidak et al., 2001). Model fitting was performed by likelihood ratio tests (LRTs) as implemented in DAMBE (Xia and Xie, 2001). A neighbour joining (NJ) phylogeny was inferred with the model selected by LRTs using MEGA2.1 (Kumar et al., 2001) and the complete gap deletion option. The robustness of the phylogeny was assessed by non-parametric bootstrapping with 1000 pseudoreplicates.

nodC amplification

Primers nodCF2 and nodCI, and amplification protocol of Laguerre et al. (2001) were used for *nodC* amplification experiments.

Statistical analysis

Data of nodulation assays were analysed using the least significant difference test according to Sendecor and Cochran (1978).

3. Results

Efficiency and host specificity of rhizobial strains

Twelve rhizobial isolates were recovered from the root nodules of *Phaseolus vulgaris* plants sampled from two regions in Egypt. Seven isolates were from the Ashmun area in the middle of the Delta-Nile Valley and five were from the Isma'ilya desert. Nodulation phenotypes for these isolates with cultivar Saxa are summarized in Table 1.

The best strain for nodule formation was EBRI 3 (129 nodules plant^{-1}) followed by EBRI 29 and CIAT 899T. Nodule fresh weight was also at its highest with strain EBRI 3, followed by EBRI 29 and EBRI 21. Plants inoculated by strain EBRI 21, 24, 3 and CIAT 899T produced the highest shoot biomass compared to control plants. Noteworthy, the weight of nodules formed by strains EBRI 3, 27 and 29 were higher than the weight of the root system after detaching the nodules (Table 1). Isolate EBRI 21 gave the highest ARA

activity with 13.7 nM C_2H_4 h^{-1} mg^{-1} nodule followed by strains EBRI 2 and EBRI 26.

Based on these results we selected the strains EBRI 2, 21 and 26 as compared to the reference strain *R. tropici* CIAT 899T to study the N_2 fixation capacity of these strains with two other cultivars, Canoca and Giza 6, at 30 days after planting (Table 2). With the cultivar Giza 6, the strains from Egypt formed the largest number of nodules and the highest nodule biomass. Strains CIAT 899T and EBRI 26 were most effective strains for nitrogen fixation. They produced 16.9 and 13.2 nM C_2H_4 h^{-1} mg^{-1} nodule. With cultivar Canoca from Colombia, the South American strain CIAT 899T of *Rhizobium tropici* gave the highest nodule biomass compared to the three strains from Egypt. With both cultivars tested in this series, only strain EBRI 2 produced consistently a nodule/root biomass ratio of under 1, whereas for the other three strains this ratio was above 1.

Table 1. Nodulation data of new isolates of bean rhizobia nodulating *Phaseolus vulgaris* cv. Saxa, 21 days after planting.

Treatment	No. of nodules plant^{-1}	FW of nodules mg plant^{-1}	FW of shoot g plant^{-1}	FW of root mg plant^{-1}	nMol C_2H_4 $h^{-1}mg^{-1}$ nodules	Ratio of nodule to root FW
Control	0	0	1.8	1,250	0	–
CIAT 899	71	578	2.4	830	6.5	0.70
EBRI 2	67	510	2.1	1,140	8.9	0.45
EBRI 3	129	907	2.4	870	3.7	1.04
EBRI 6	0	0	1.9	780	0	–
EBRI 20	0	0	1.8	260	0	–
EBRI 21	73	695	2.6	1,160	13.7	0.56
EBRI 23	0	0	1.7	340	0.0	–
EBRI 24	60	670	2.5	690	7.5	0.97
EBRI 25	0	0	1.9	900	0	–
EBRI 26	62	507	2.4	930	7.9	0.55
EBRI 27	67	561	2.2	350	6.2	1.60
EBRI 29	79	715	2.2	290	6.4	2.46
EBRI 32	52	600	2.0	650	Nd	0.92
L.S.D 0.05	27.8	281	Ns	240	3.82	–

Results are taken from three replicates for each parameter. EBRI: Egyptian bean rhizobial isolates. Ns: non significant result. Nd: not determined.

Table 2. Nodulation data of selected isolates of bean rhizobia with different host cultivars of *P. vulgaris*, 30 days after planting.

Treatment	No. of nodules plant^{-1}	FW of nodules mg plant^{-1}	FW of shoot g plant^{-1}	FW of root mg plant^{-1}	nMol C_2H_4 h^{-1}mg^{-1} nodules	Ratio of nodule to root FW
Giza 6						
Control	0	0	5.1	2,050	0	–
CIAT 899	132	812	9.8	390	16.7	2.1
EBRI 2	192	1,692	14.4	2,300	7.1	0.73
EBRI 21	170	991	12.3	890	9.5	1.11
EBRI 26	165	896	10.8	800	13.2	1.12
Canoca						
Control	0	0	2.3	1,390	0	–
CIAT 899	153	1,289	10.1	800	9.4	1.61
EBRI 2	91	838	8.3	1,310	14.3	0.63
EBRI 21	161	772	9.0	490	7.6	1.58
EBRI 26	203	948	8.3	790	12.7	1.20
L.S.D 0.05	62.91	460	3.57	530	5.02	–

Molecular characterization

Amplified ribosomal DNA restriction analysis (ARDRA) of 16S and 23S rDNA fragments, and phenotypic characteristics

All Egyptian isolates and standard strains yielded *rrs* amplification products of about 1,500 bp and *rrl* amplicons of about 2,100 bp. The *rrs* amplicons were subjected to digestion with enzymes *Hha1*, *Dde1*, *Msp1* and *Sau3a1* while the products from the locus *rrl* were restricted with *Hinf1*, *Msp1* and *Sau3a1*. A Dice/UPGMA analysis of the combined restriction patterns from the 16S and 23S amplicons resulted in the dendrogram shown in Fig. 1.

Three major genotypes (A, B and C) were identified among the 12 Egyptian bean isolates. Strains with genotype A (EBRI 2, 3, 21, 24, 26, 27 and 29) formed the predominant group which was related to *R. etli* CFN42T. These strains were able to grow on MM medium with malate as a sole carbon source but were unable to grow on the same medium with sorbitol as a main carbon source (Table 3). Similar results were reported by Segovia et al. (1993). Strain EBRI 32, which nodulated *Phaseolus vulgaris* (Table 1) and *Leucaena leucocephala* (Table 4) had 16S and 23S rDNA ARDRA patterns identical to those from *R. gallicum* R602spT, forming group B.

Table 3. Phenotypic characteristics of rhizobial strains on different media.

Strain	Growth on LB medium	Growth on MM medium with		Growth on basal medium with	
		Malate	Sorbitol	D-Glucuronate	5 μg ml^{-1} spectinomycin
R. etli CFN42T	–	+	–	+	–
EBRI 2	–	+	–	+	+
EBRI 3	–	+	–	+	–
EBRI 6	+	+	+	+	+
EBRI 20	+	+	+	+	+
EBRI 21	–	+	–	–	–
EBRI 23	+	+	+	+	+
EBRI 24	–	+	–	+	+
EBRI 25	+	+	+	+	+
EBRI 26	–	+	–	+	+
EBRI 27	–	+	–	–	–
EBRI 29	–	+	–	–	+
EBRI 32	–	+	+	–	–
R. gallicum R602spT	–	+	+	–	–
R. tropici CIAT899T	+	+	+	+	+
R. giardinii H152T	–	+	+	+	+

+: positive growth. –: no growth.

Strain EBRI 32 was not able to grow on basal medium containing glucuronic acid as a sole carbon source or with 5 μg ml^{-1} spectinomycin, and was not able to grow on LB medium (Table 3) as previously mentioned for *R. gallicum* (Amarger et al., 1997). Finally, isolates EBRI 6, 20, 23 and 25 presented RFLP patterns highly related to those from *A. tumefaciens* DSM 30150, forming group C. These four strains failed to re-nodulate *Phaseolus vulgaris* cultivar Saxa (Table 1) and were able to survive on LB medium as reported by Amarger et al. (1997).

Plasmid DNA content

Fourteen plasmid patterns were identified among the 16 rhizobial strains tested (Table 4). Plasmid numbers varied from 2 to 6 and their sizes ranged from 40 kb to 650 kb. All strains grouped in the ARDRA cluster A contained a large plasmid of about 650 kb, whereas the largest plasmid found in strains from

Table 4. Nodulation of legume tree and plasmid DNA content of rhizobial strains used in this study.

Strain	Nodulated *L. leucocephala*	Size of plasmid kb	ARDRA cluster
R. etli CFN42T	–	650–510–390–270–180–150	
EBRI 2	–	650–510–400–390–260–65	A
EBRI 3	–	650–400–390–180	A
EBRI 6	–	250–140–108	C
EBRI 20	–	150–65–45	C
EBRI 21	–	650–490–250	A
EBRI 23	–	270–115–65–45	C
EBRI 24	–	650–510–390–250–190	A
EBRI 27			
EBRI 25	–	380–250–40	C
EBRI 26	–	650–510–390–250–208	A
EBRI 29	–	>1.500–650–400–390–180	A
EBRI 32	+	550–250	B
R. gallicum R602spT			
R. tropici CIAT899T	+	>1.500–550–250	
R. giardinii H152T	+	140–120	

+: able to nodulate *L. leucocephala*. –: unable to nodulate *L. leucocephala.*

cluster B was 550 kb, and that found in strains from cluster C only 380 kb. Plasmid profile analysis provided higher taxonomic resolution than the RFLP analysis. Each strain had a unique plasmid profile except for the pair EBRI 24 and EBRI 27, which shared the same plasmid patterns. Irrespective of the megaplasmids, strain EBRI 32 displayed the same plasmid profile as strains *R. tropici* CIAT 899T and *R. gallicum* R602spT. It should be noted that the megaplasmids present in the two later strains (Silva et al., 2003) could be detected only for *R. tropici* CIAT 899T and *R. etli* EBRI 29 but could not be resolved for *R. gallicum* R602spT in our gels.

Phylogenetic analysis of rrs sequences

Fig. 2 shows the phylogenetic placement of eight Egyptian bean nodule isolates in the context of a neighbour-joining (NJ) phylogeny inferred from the *rrs* sequences of all rhizobial type strains and those from closely related non-rhizobial species. Due to the bias in base composition and transitional substitutions present in the data set, the Tamura-Nei (TrN93), a model of nucleotide substitution was used to reconstruct the phylogeny, which provided a highly significant ($P<0.000001$; DF=2) improvement in the fit of the model to

the data ($-LnL_{TrN93}$=7509.9307) over the Jukes-Cantor (JC) model (LnL_{JC}=7615.3545).

Model fitting was further improved with very high statistical significance (LRT= 212.0586; P<0.00001) by accounting for among-site rate variation using a discrete gamma distribution with 8 rate categories, with an estimated shape parameter of value α=0.15 (-LnLTrN+G=7403.9014). Therefore, the NJ phylogeny shown in Fig. 2 was reconstructed using the TrN+G model. This phylogeny confirms the ARDRA results. Strains EBRI 2, 3, 21, 26 and 27 form a highly supported clade along with *R. etli* CFN 42T. Strains EBRI 20 and 25 are significantly associated to the lineages of *R. radiobacter* and *R. rubi* (formally *A. radiobacter* and *A. rubi*), respectively.

PCR amplification of nodC gene fragments

All strains yielded *nodC* amplification products of about 1,450 bp except the *Agrobacterium*-like isolates (data not shown). Seven strains yielded two fragments, while strains EBRI 21, 27, R602sp and H152 produced only one fragment.

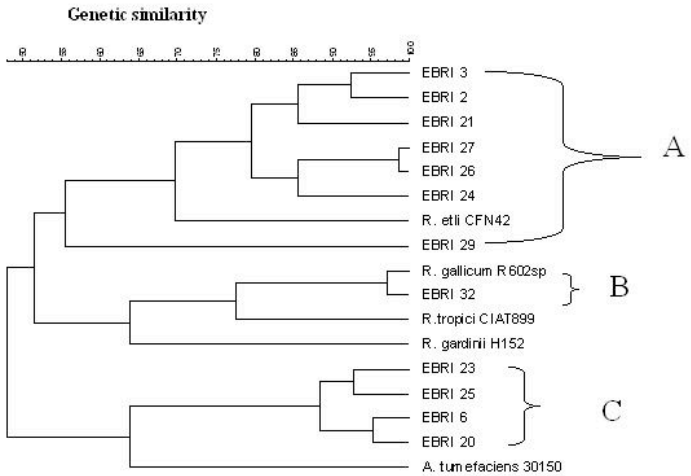

Figure 1

Figure 1. DICE/UPGMA analysis of 16S and 23S rDNA amplicons from twelve Egyptian isolates and 5 reference strains. The 16S rDNA amplification products were digested with enzymes *Hha1*, *Dde1*, *Msp1* and *Sau3a1*, and the 23S rDNA amplicons with *Hinf1*, *Msp1* and *Sau3a1*.

rrs phylogeny
NJ-TrN+G
α= 0.15
1000 bootstrap replicates
52 taxa
1276 sites, complete gap deletion

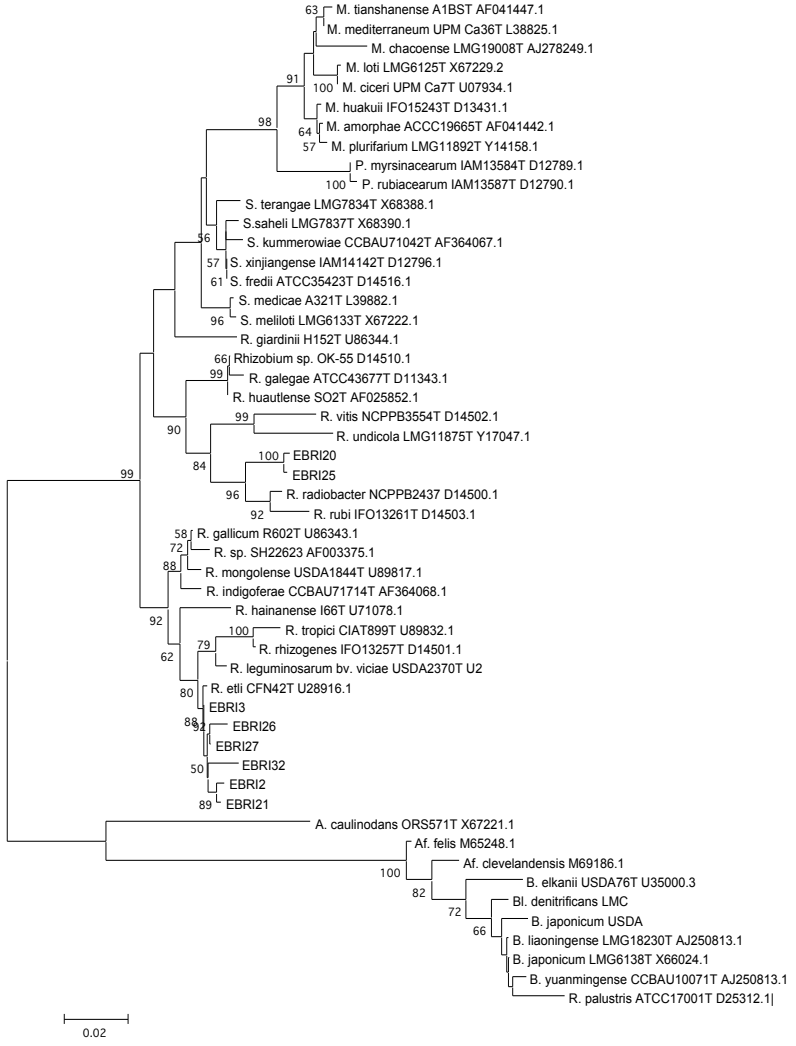

Figure 2. Neighbour-joining phylogram (TrN93+G, α=0.15) based on 1,300 bp of aligned *rrs* sequences from 6 Egyptian and 44 reference strains. Bootstrap support values greater than 50% (out of 1000 pseudoreplicates) are indicated at the branching points. Numbers on the left side are the accession numbers for the sequences used.

Similar results were reported by Mhamdi et al. (2002). ARDRA, 16S rDNA phylogeny, nodulation assays and plasmid profiling experiments consistently indicated that isolates EBRI 6, 20, 23 and 25 were closely related to the *A. radiobacter* lineages. These were the only strains that did not yield *nodC* amplification products, suggesting that these are not symbiotic isolates, which is consistent with their inability to nodulate *P. vulgaris* (Table 1).

4. Discussion

Phaseolus vulgaris nodules can fix about 40–50% of the plant's N-demand by symbiotic N_2 fixation (Werner, 1992), but efficiencies in the field are often much lower. This is partly due to the relatively broad host-range symbiotic association exhibited by common bean, which is known to nodulate in nature with at least five *Rhizobium* species of contrasting symbiotic competitiveness and efficiency (Martinez-Romero, 2003; Mhamdi et al., 1999; Silva et al., 1999; Silva et al., 2003). It is therefore advantageous to select effective strains for specific cultivars from the region where the beans are grown. To achieve this goal, 12 strains were isolated from two geographic regions of Egypt to characterize them taxonomically as well as to evaluate some of their key symbiotic phenotypes.

The genetic diversity of the EBRI isolates was first analysed by combined 16S and 23S ARDRA, which revealed that most of the isolates (58%) were closely related to *R. etli*. Four isolates were related to *A. tumefaciens* (*R. radiobacter*), whereas only one isolate was related to *R. gallicum*. This was confirmed by phylogenetic analysis of nearly full-length 16S rDNA sequences, the key gene used in current rhizobial systematics for species identification (Sawada et al., 2003). Strain EBRI 32, which was grouped with *R. gallicum* in the ARDRA, had a nodulation phenotype and a plasmid profile similar to those exhibited in *R. gallicum* R602sp but clustered with the genetic lineage of *R. etli* (Fig. 2) suggesting a possible gene transfer, causing heterogeneity in *rrs* phylogeny (Ueda et al., 1999). Plasmid profiles also differentiated these three major lineages of isolates, as did discriminatory phenotypic characters such as growth on LB medium, growth on basal medium with glucuronic acid as a main carbon source or addition of 5 μg ml^{-1} spectinomycin, growth on MM medium with malate or sorbitol as a carbon source, colony morphology and nodulation parameters.

Based on these data we can conclude, that the isolates analysed in this study correspond to *R. etli*, *R. gallicum* and a *Rhizobium* lineage closely related to *R. radiobacter* (former *A. tumefaciens*). These results are consistent with those reported for bean nodule isolates from Tunisia, (Mhamdi et al., 1999; 2002) and from Central and West Africa (Diouf et al., 2000) who found that *R. etli* is the

predominant common bean micro-symbiont in these soils. Although the latter authors did not find *Agrobacterium*-like isolates, others have isolated such bacteria from diverse hosts, including common bean nodules from Morocco (Drevon et al., 2001), from Tunisia (Mhamdi et al., 2002), from legume nodules from Pakistan (Hameed et al., 2004) and nodules from tropical legumes (de Lajudie et al., 1999). The *Agrobacterium*-like isolates seem to be symbiotically unstable under laboratory conditions, loosing the ability to nodulate the host, which is likely due to loss of key symbiotic genes or the entire pSym as evidenced by our *nodC* PCR ampilification and plasmid profiling experiments.

The symbiotic instability of *Agrobacterium*-like isolates has been observed by other workers (de Lajudie et al., 1999; Martinez et al., 1987) and this may be the most probable explanation for the loss of symbiotic phenotype of isolates EBRI 6, 20, 23 and 25. Such isolates are therefore a poor choice for the formulation of common bean inoculants. Hungria and Araujo (1995) reported that the strain SEMIA 4064, used as a commercial inoculant lost its ability to fix nitrogen with common bean under field and green house conditions. These observations indicate that inoculation programs should be directed not only to select effective rhizobial strains but also to select strains have genetic stability to avoid to lose the pSym plasmid or genomic rearrangements (Flores et al., 2000).

The initial nodulation experiments using the German *P. vulgaris* cv. Saxa germplasm, the Colombian cultivar Canoca and the Egyptian cultivar Giza 6 revealed that there is remarkable degree of host-preference for each strain. Nodulation experiments with *Leucaena leucocephala* cv. Cunningham, indicate that only strain (EBRI 32) was able to nodulate this tree legume and failed to grow on LB medium, which is consistent with its classification as *R. gallicum* (Amarger et al., 1997).

In conclusion, effective *R. etli* and *R. gallicum* isolates were obtained from field grown common bean nodules in Egypt, which exhibited contrasting host-dependent symbiotic efficiencies. Our next studies will focus on nodulation competitiveness with the ultimate goal of developing a multi-strain inoculum for *P. vulgaris* in Egyptian soil.

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High Salt and High pH Tolerance of New Isolated *Rhizobium etli* Strains from Egyptian Soils

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Abstract. Saline and alkaline soils are major problems contributing to the low productivity of common bean (*Phaseolus vulgaris*) in arid and semi-arid regions such as Egypt. Therefore our study was directed toward selecting strains more tolerant to these environmental stresses. Among seven *Rhizobium etli* strains isolated from Egyptian soils, we found a high degree of diversity. Strains EBRI 21 and EBRI 26 are highly tolerant to a salt concentration up to 4% NaCl. A positive correlation was found between the salt tolerance and the adaptation to alkaline pH (9). Strains EBRI 2 and EBRI 26 were adapted to elevated temperatures (42°C). The minimum level of low pH for the majority of *Rhizobium etli* strains from Egypt was pH 4.7 while the Colombian strain *Rhizobium tropici* CIAT 899 survived well at pH 4. At 0.4% NaCl, the symbiotic efficiency of the salt-tolerant strain EBRI 26 was superior in cultivar Giza 6 compared with the salt-sensitive strain EBRI 2 (18.2 compared with 13.9 nM C_2H_4 h^{-1} mg^{-1} nodule fresh weight). In the bean cultivar Saxa, nitrogen fixation was much more affected by high salt concentration (0.4% NaCl) than in the cultivar Giza 6 with both strains (3.9 and 3.8 nM C_2H_4 h^{-1} mg^{-1} nodule fresh weight respectively). In general, stress of alkalinity had a less detrimental effect on nodulation and N_2 fixation than stress of salinity.

Nearly 10% of the world's land surface can be classified as endangered by salinity. Most such areas are in the tropics and the Mediterranean regions [16]. Arid land in Egypt represents 97% of the total area, characterized by high temperature, low relative humidity, high rate of evaporation, and little rainfall, leading to degraded soils [20]. Legume plants are more sensitive to salinity than their rhizobial partners. Consequently, symbiosis is more sensitive to salt stress than free-living rhizobia [20]. There is some evidence that rhizobial strains isolated from alkaline soils are rather tolerant to high temperature, pH, and salt stress [16]. An efficient *Rhizobium*–legume symbiosis under salt stress requires also the selection of salt-tolerant rhizobia [20]. The effects of salt or pH stress on nitrogen fixation have been reported in several studies [3, 4, 8, 13, 16, 20]. Therefore our work focused on: (a) selection of new rhizobial strains adapted to high salt and pH ecosystems, and (b) studying the symbiotic performance of these strains under these conditions.

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Materials and Methods

Bacterial strains. Seven *R. etli* strains, EBRI 2, 3, 21, 24, 26, 27, and 29, were isolated from nodules of common bean grown in two different locations of the Nile Valley Delta and the Isma'ilya desert soil in Egypt. Roots of common bean plants were collected with a block of soil and transferred to the laboratory for rhizobial isolation. Rhizobial strains were isolated from surface-sterilized nodules according to the method described by Somasegaran and Hoben [14]. Strains were cultivated in 20E medium [19] at 28°C for 48 h. *R. tropici* strain CIAT 899 was obtained from the culture collection of the Cell Biology and Applied Botany Department at Marburg University. Strains were kept in 50% glycerol at −70°C for further studies.

Selection of strains adapted to environmental stresses. Growth of *R. etli* strains and strain *R. tropici* CIAT 899 was tested in 20E medium

amended with different NaCl concentrations to select strains tolerant to saline soils [9]. To obtain strains resistant to alkaline conditions (pH > 7–9) the medium was buffered with AMPD buffer, while for the low pH range between 4 and 5 it was buffered with 25 mM HOMOPIPES. To adjust the medium pH from 5.5 to 7.20 mM MES was added as described by Priefer et al. [12]. To test temperature tolerance, rhizobial cultures on a roller-shaker were incubated inside an incubator with the temperature adjusted to 37°C or 42°C compared with the optimum of 30°C. The growth was recorded as optical density (OD) with three replicates.

Plant nodulation assays and symbiotic performance under stress of salinity or alkalinity. The nodulation assays were performed in sterilized Leonard jars with vermiculite: perlite (1:1 v:v) and N-free nutrient solution [19]. Seeds of the common bean cultivars Saxa and Giza 6, from Germany and Egypt respectively, were surface-sterilized [18] and distributed on the surface of 1% agar plates, and incubated for 3–4 days at 28°C for germination. After germination, seedlings were transferred to Leopard jars. Seeds were inoculated by adding 1 mL of rhizobial cultures at the middle of the exponential growth phase, with 10^8 cells ml^{-1} for each Leonard jar. Plants were cultivated in a controlled growth chamber with 15 h of light at 25°C and 9 h of darkness at 18°C, relative humidity of 75% and light intensity of 14 klux. To study the symbiotic performance of strains under stress of salinity, sodium chloride was added to the nutrient solution at a final concentration of 0.2% NaCl (34.2 mM) or 0.4% NaCl (68.4 mM). For alkaline stress, the nutrient solution was buffered with 20 mM AMPD to pH 8 and pH 8.5 [12]. Each treatment was replicated three times. Plants were harvested 21 days after inoculation. Acetylene reduction assay (ARA) was estimated as described by Bender and Rolfe [2], and nodulation parameters of nodule number, nodule fresh weight, and shoot fresh weight were recorded.

Results and Discussion

Selection of strains well adapted to environmental stresses. Soil degradation due to salinization or alkalization is one of the most serious problems affecting the fertility of soils, especially in arid and semi-arid areas. Ten percent of the total degraded soils in the world are high-saline or high-pH soils [16]. Consequently, we aimed to test new isolates of *R. etli* as regards their tolerance to high salt, pH and temperature, to select strains resistant to these environmental conditions. The results shown in Table 1 demonstrate that among eight strains we found a high degree of diversity. Two strains were highly tolerant to a salt concentration up to 4% NaCl (EBRI 21 and EBRI 26) and three strains were moderately tolerant up to 3% NaCl (EBRI 24, 27, and 29). These data are consistent with the results obtained by Graham and Parker [6] showing that strains of fast-growing acid-producing rhizobia such as *R. etli* were generally more salt-tolerant than slow-growing alkali-producing strains. Strains EBRI 2 and EBRI 3 were the most sensitive to salt concentration. Their growth was already suppressed by the addition of 0.6% NaCl. Amarger et al. [1] noted that tolerance to salinity, acidity, and alkalinity was more strain-specific than species-specific. Strain *R. tropici* CIAT 899 was able to resist NaCl concentrations up to 2.5%. This strain could grow at 4% NaCl only when the incubation time was prolonged to 6 days (data not shown). Similar results have been reported by Nogales et al. [10]. Strains tested in this study also seemed to be well adapted to high or low pH. At low pH, strains EBRI 2, 21, 26, 27, and 29 were better adapted than strains EBRI 3 and 24. Strain *R. tropici* CIAT 899, isolated from acid soils in Colombia, was reported to be more tolerant to low pH [15, 17] than the Egyptian *R. etli* strains. At high pH, strains EBRI 2, 3, and 27 were less tolerant than strains EBRI 21, 24, 26, and 29. All strains tolerating salt concentrations from 2% to 4% NaCl were highly resistant to alkaline conditions except strain EBRI 27 (Table 1). A significant positive correlation was found between the salt tolerance and the adaptation of rhizobial strains in alkaline conditions. These findings confirm previous reports with *Rhizobium* strains from other areas [8].

Table 1. Phenotypic characteristics of *Rhizobium etli* strains nodulating *Phaseolus vulgaris* from Egypt grown under environmental stresses

Strain	NaCl % inhibiting the growth	Low pH values tolerated	High pH values tolerated	Maximal temperature tolerated (°C)
R. tropici				
CIAT 899	3	4	9	37
R. etli strains				
EBRI 2	0.6	4.7	8.5	42
EBRI 3	0.6	5.5	8.5	37
EBRI 21	4.5	4.7	9	30
EBRI 24	3.5	5.5	9	37
EBRI 26	4.5	4.7	9	42
EBRI 27	3.5	4.7	8.5	37
EBRI 29	3.5	4.7	9	37

EBRI, Egyptian Bean Rhizobial Isolates.

Nodule functions in *Phaseolus vulgaris* are hampered by a root temperature above 30°C [11]. To identify strains tolerant to high temperatures, strains were grown at 37°C or 42°C in 20E medium. Strains EBRI 2 and EBRI 26 grew still at 42°C. Karanja and Wood [7] have isolated bean rhizobial strains grown at 45°C. Strains EBRI 3, 24, 27, and 29 were able to survive at 30°C. Strain EBRI 21 was able to survive only at the optimum temperature.

Effect of interaction between high salt concentrations and high pH levels on the growth of rhizobial strains. Strains EBRI 21 and EBRI 26 (salt-tolerant) and strain EBRI 2 (salt-sensitive) were selected to study the interaction between salinity and alkalinity; for

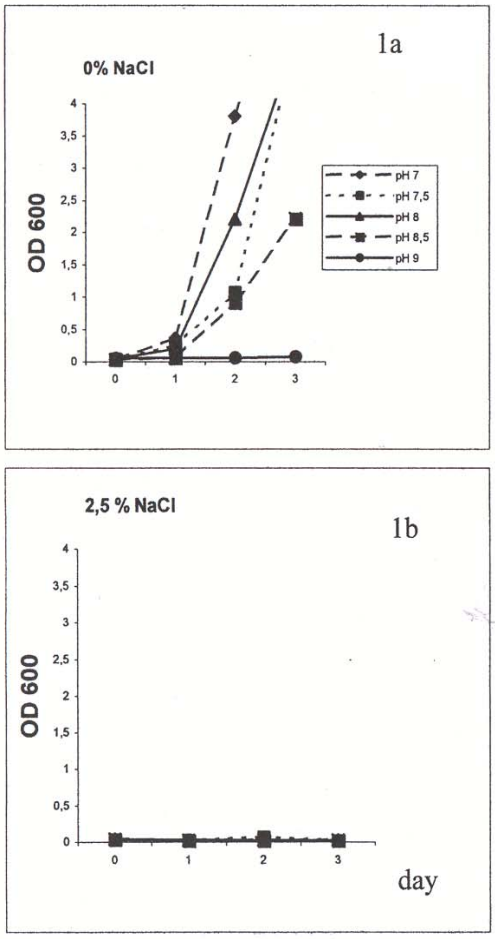


Fig. 1. Growth curves of *Rhizobium etli* EBRI 2 at different pHs and salinity levels.

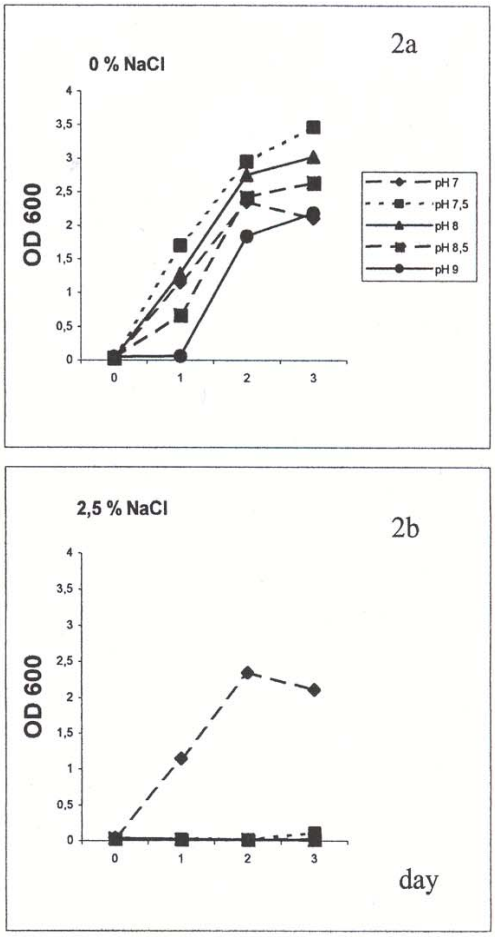


Fig. 2. Growth curves of *Rhizobium tropici* CIAT 899 at different pHs and salinity levels.

comparison we tested strain *R. tropici* CIAT 899. The interaction between high salt and high pH on the viability of *R. etli* strain EBRI 2 was evaluated after 3 days. This strain was able to survive at different levels of pH from 7.5 to 9 (Fig. 1a), as compared with pH 7 without salt. With 2.5% NaCl (Fig. 1b), no growth was observed at all levels. The standard type strain *Rhizobium tropici* CIAT 899 was less tolerant to the combinations of salt and alkaline stress than the Egyptian isolates. It was able to survive only at 2.5% NaCl and pH 7 (Fig. 2b) compared with the ability of this strain to grow at different pH levels without salinity (Fig. 2a). The salt-resistant strain EBRI 21 survived well (Fig. 3b, c) with 2.5% and 3% NaCl at different pH levels. Strain EBRI 26 had the same physiological behavior as EBRI 21 under stress of salt and alkaline conditions (Fig. 4a–c). The results are in agreement with those obtained by Kulkarni et al. [8]. Both salt-tolerant strains (EBRI 21 and 26) were unable to grow at 4% NaCl plus different pHs, although they could survive at 4% NaCl without the stress of alkalinity (Table 1).

Nodulation parameters and N_2 fixation under stress of salinity or alkalinity. The symbiotic performance of EBRI 2 and EBRI 26 as a salt-sensitive and salt-resistant strain respectively was determined under high salt or high pH with two cultivars of common bean. Results in Table 2 summarize the nodulation parameters of strains EBRI 2 and EBRI 26 under stress of salinity with cultivars Giza 6 and Saxa. In cultivar Giza 6, no significant difference was observed between the nodule numbers formed by the salt-sensitive strain EBRI 2 without salt and under stress of salt (0.2% NaCl). At 0.4% NaCl, the process of nodule formation in *Phaseolus*

Table 2. Nodulation parameters of selected *Rhizobium etli* strains with two host cultivars under stress of salinity, 21 days after infection

Treatments	No. of nodules per plant	(FW) of nodules (mg plant^{-1})	FW of shoot (g plant^{-1})	nmol C_2H_4 h^{-1} mg^{-1} nodules (FW)
Plant cultivar Giza 6				
Control	0.0^{c}	0.0^{d}	3.4^{c}	0.0^{f}
Inoculated with EBRI 2	103^{ab}	309^{ab}	4.3^{b}	29.9^{a}
Inoculated with EBRI 2 + 0.2% NaCl	123^{a}	374^{a}	5.4^{a}	24.6^{b}
Inoculated with EBRI 2 + 0.4% NaCl	56^{ba}	150^{cb}	4.3^{b}	13.9^{dc}
Inoculated with EBRI 26	54^{ba}	163^{cb}	4.6^{b}	26.8^{a}
Inoculated with EBRI 26 + 0.2% NaCl	108^{ab}	335^{ab}	5.6^{a}	24.1^{b}
Inoculated with EBRI 26 + 0.4% NaCl	83^{ba}	224^{ba}	4.7^{b}	18.2^{c}
Plant cultivar Saxa				
Control	0.00^{c}	0.00^{d}	1.9^{d}	0.0^{f}
Inoculated with EBRI 2	81^{ba}	239^{ba}	3.0^{c}	18.2^{c}
Inoculated with EBRI 2 + 0.2% NaCl	88^{ab}	344^{ab}	3.1^{c}	15.7^{cd}
Inoculated with EBRI 2 + 0.4% NaCl	61^{ba}	160^{cb}	2.2^{d}	3.8^{e}
Inoculated with EBRI 26	66^{ba}	267^{ba}	3.3^{c}	19.8^{c}
Inoculated with EBRI 26 + 0.2% NaCl	113^{ab}	357^{ab}	2.9^{c}	17.4^{c}
Inoculated with EBRI 26 + 0.4% NaCl	89^{ab}	224^{ba}	2.6^{c}	3.9^{e}
LSD 0.05	**37.31**	**96.74**	**0.59**	**3.56**

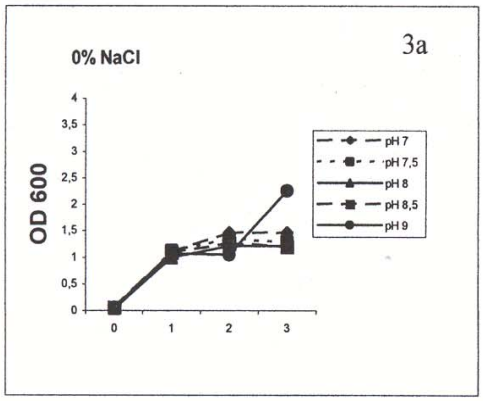
FW, fresh weight.
Letters on the right side indicating on the statistical analysis.

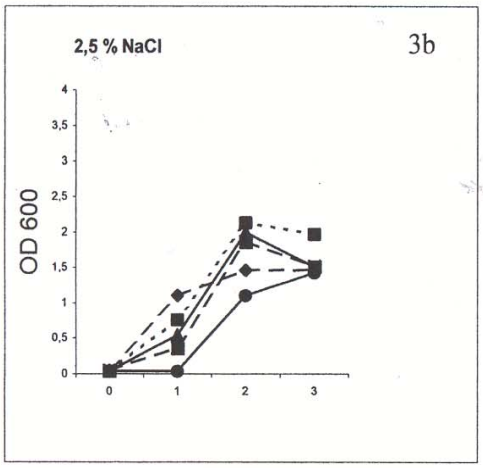
Table 3. Nodulation and nitrogen fixation of selected *Rhizobium etli* strains under stress of alkalinity with two host cultivars, 21 days after infection

Treatments	No. of nodules per plant	FW of nodules (mg plant^{-1})	FW of shoot (g plant^{-1})	nmol C_2H_4 h^{-1} mg^{-1} nodules (FW)
Plant cultivar Giza 6				
Control	0.0^{c}	0.0^{d}	3.00^{b}	0.0^{c}
Inoculated with EBRI 2	70^{a}	147^{cb}	4.4^{a}	14.9^{b}
Inoculated with EBRI 2 + pH 8	67^{ba}	189^{b}	4.1^{a}	24.2^{a}
Inoculated with EBRI 2 + pH 8.5	50^{ba}	127^{cb}	3.6^{ba}	19.3^{ba}
Inoculated with EBRI 26	69^{a}	188^{b}	4.4^{a}	23.7^{a}
Inoculated with EBRI 26 + pH 8	67^{ba}	206^{b}	4.8^{a}	27.2^{a}
Inoculated with EBRI 26 + pH 8.5	52^{ba}	181^{b}	3.7^{ba}	24.9^{a}
Plant cultivar Saxa				
Control	0.0^{c}	0.0^{d}	2.1^{c}	0.0^{c}
Inoculated with EBRI 2	70^{a}	177^{b}	2.4^{ba} bc	12.8^{b}
Inoculated with EBRI 2 + pH 8	91^{a}	253^{a}	2.8^{b}	11.6^{b}
Inoculated with EBRI 2 + pH 8.5	63^{ba}	170^{b}	2.8^{bc}	20.4^{ba}
Inoculated with EBRI 26	79^{a}	292^{a}	2.8^{bc}	16.2^{b}
Inoculated with EBRI 26 + pH 8	72^{a}	200^{b}	2.5^{bc}	18.8^{ba}
Inoculated with EBRI 26 + pH 8.5	59^{ba}	152^{b}	2.7^{bc}	25.7^{a}
LSD 0.05	**21.35**	**42.72**	**0.69**	**5.79**

FW, fresh weight.
Letters on the right side indicating on the statistical analysis.

vulagris was reported to be extremely sensitive to NaCl. A reduction in nodule number, nodule fresh weight, and acetylene reduction by 45%, 51%, and 53% respectively was observed with the salt-sensitive strain EBRI 2. Saadallah et al. [13] noticed that the symbiotic interaction between *P. vulgaris* and its micro-symbionts was negatively affected by salt concentration of 0.3% NaCl. Cordovilla et al. [3] found a reduction in nodule number and nodule weight in faba bean by factor 45% and 59% respectively at 0.4% NaCl. With the salt-





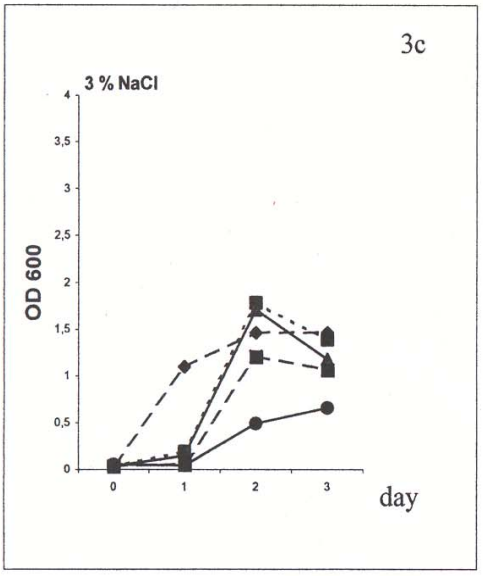
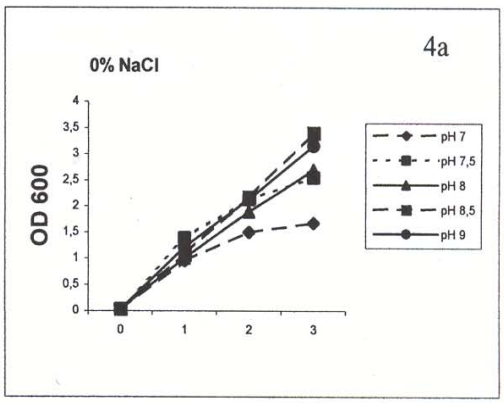
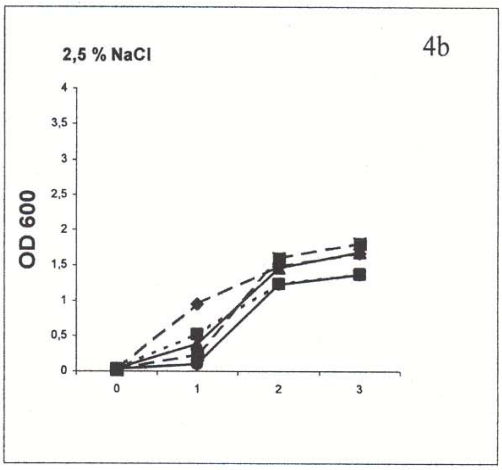


Fig. 3. Growth curves of *Rhizobium etli* EBRI 21 at different pHs and salinity levels.





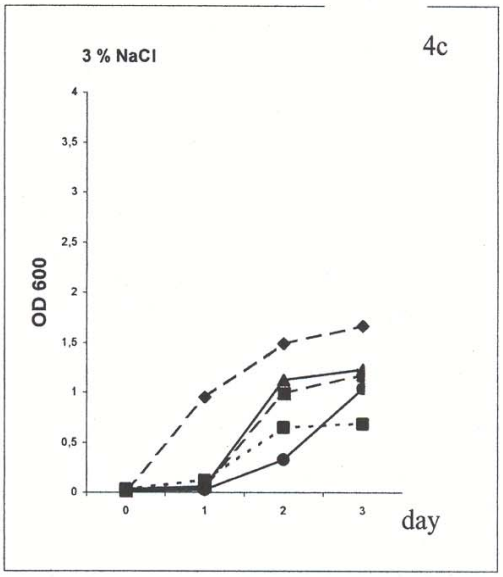


Fig. 4. Growth curves of *Rhizobium etli* EBRI 26 at different pHs and salinity levels.

resistant strain EBRI 26, the addition of sodium chloride to the nutrient solution promoted the formation of nodules. Our results also confirm the findings of Elsheikh and Wood [4] with soybeans. A major criticism of these reports is that the difference in symbiotic efficiency may be due to genotypic variations among legume cultivars not to the salt-tolerant rhizobial strains [20]. In our studies with cultivar Saxa, no effect was observed in nodulation, but the nitrogenase activity was strongly depressed at 0.4% NaCl with the salt-sensitive EBRI 2 as well as with the salt-resistant EBRI 26 (3.9 and 3.8 nM C_2H_4 h^{-1} mg^{-1} nodules). No such effect was observed with the cultivar Giza 6 from Egypt. Saadallah et al. [13] found a genotypic variation among nine common bean cultivars in their tolerance to salt. Our results indicate that there is a specific cultivar-dependent salt effect on nitrogen fixation, not visible in the formation of nodule mass. Results in Table 3 show the symbiotic efficiency of strains EBRI 2 and EBRI 26 under alkaline conditions. Both strains EBRI 2 and EBRI 26 were able to nodulate and fix nitrogen with cultivars Giza 6 and Saxa at pH 8 and pH 8.5. Based on the parameters of nodule number and nodule fresh weight, stress of high pH has less detrimental effects on nodule formation and also nitrogen fixation compared with salinity tests (Table 2). Similar results have been reported by Evans et al. [5], who found that nodule formation increased in alkaline soils. Surprisingly, the increasing in nitrogen fixation per nodule fresh weight was observed in cultivars under high alkaline conditions compared with neutral pH. Future studies are needed to select the salt-tolerant strain of *R. etli* EBRI 26 as model strain, in order to study the genes involved in salt tolerance and the relationship between these genes and the symbiotic genes.

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