Genetic Variation of *Sinorhizobium meliloti* Isolates Differing in Their Ability to Drought Tolerance

الاختلافات الوراثية بين عزلات *Sinorhizobium meliloti* في قابليتها على تحمل الجفاف

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Abstract

Soil bacteria *Sinorhizobium meliloti* had enormous agricultural value, due to their ability in fixing nitrogen symbiotically with an important forage crop legume- alfalfa. The aim of this study (i) isolate indigenous S. *meliloti* from different field sites in Iraq, (ii) evaluate the isolates tolerance to induce drought using polyethylene glycol-6000, (iii) assessing genetic diversity and genetic relationships among isolates of natural population with drought tolerant abilities. Drought tolerance study revealed vast variations between *Sinorhizobium* isolates, the highest tolerant isolates to drought were twelve from total thirty 40%, tolerated from -3 up to -4 Mpa (mega pascal), while the drought sensitive isolates tolerated upto – 1.5 Mpa, except isolate Bs58 which tolerated upto -1 Mpa water potential. The growth declined with the increase of drought stress. Cluster analysis based on RAPD-PCR showed significant differences among *S. meliloti* isolates, and the results gave almost identical grouping of isolates in regards to drought experiment. Among indigenous isolates two divergent groups could be determined, the first major group included drought tolerant isolates and the second major group comprised all drought moderate and sensitive isolates with 40% similarity between the two major groups.

Key words: *Sinorhizobium meliloti*, RAPD, drought, genetic diversity.

الملخص

بكتريا التربة *Sinorhizobium meliloti* لها اهمية كبيرة في الزراعة وذلك لقابليتها على تثبيت النتروجين تعايشيا مع نبات الجت البقولي. الهدف من الدراسة :1) الحصول على عزلات من *Sinorhizobium* من مناطق مختلفة من العراق 2) تقييم تحمل العزلات للجفاف المستحث بواسطة البولي اثيلين كلايكول6000 ,3) تحديد التغايرات والعلاقات الوراثية بين العزلات بناءا على قابليتها لتحمل الجفاف. ان دراسة تحمل الجفاف بينت وجود تغايرات كثيرة بين عزلات *Sinorhizobium* وبلغت العزلات المتحملة اثنى عشر من مجموع ثلاثين عزلة 40% حيث تحملت من -3 الى -4 ميغاباسكال بينما العزلات الحساسة للجفاف تحملت 1 , 5 ميغاباسكال باستثناء العزلة Bs 58 تحملت لغاية -1 ميغاباسكال جهد مائي. النمو انخفض بزيادة الجفاف كما ان تحليل شجرة العلاقة بين العزلات بناءا على نتائج التضخيم العشوائي للدنا بجهاز البلمرة الحراري اظهرت وجود اختلافات واضحة بين عزلات *Sinorhizobium meliloti* وهذه النتائج جاءت متوافقة مع نتائج تجربة تحمل الجفاف حيث قسمت العزلات الى مجموعتين رئيسية الاولى ضمت جميع العزلات المتحملة للجفاف والثانية ضمت العزلات الحساسة والمتوسطة التحمل للجفاف وبنسبة تشابهه بلغت 40%بين المجموعتين.

الكلمات المفتاحية: *Sinorhizobium meliloti* ، جفاف، تغاير وراثي، RAPD

Introduction

Dry lands covers 40% of the worlds land surface and serve as the habitat and surface of livelihood for more than 1 billion people [1]. Alfalfa (*Medicago sativa*) and its symbiont *Sinorhizobium meliloti* have long history of co existence and co evolution. The natural nodulating populations of *S. meliloti* plays a major role satisfying the nitrogen requirements of the plants especially after the reduced need for application of nitrogenous fertilizers. Rhizobia had a great agricultural value in improving soil fertility in farming systems [2]. The population of soil bacteria decreases along with water stress, drought effect on rhizobia causes dehydration and change cell morphology [3]. An essential aspect of the strategy to improve the yield of arid legumes in stressed environments must involve a combination of stress –tolerant cultivars and stress- tolerant rhizobia. Various phenotypic and genotypic techniques were used to identify and characterize bacteria. Although phenotypic methods are more reliable and authenticated for identification and to study genetic diversity of bacterial isolates [4,5]. A several

number of molecular methods based on polymerase chain reaction have proposed to characterize *Sinorhizobium* strains and to provide a high degree of differentiation among closely related bacterial strains. The random amplified polymorphic DNA (RAPD) finger-printing method is based on the use of short primers which hybridize with sufficient affinity chromosomal DNA sequences at low annealing temperature in a way that they can be used to initiate amplification of regions of the bacterial genome [6].

The number and location of these random sites vary for different strains of bacterial species. RAPD – PCR has been proposed for identification and phylogenetic grouping of *Sinorhizobium meliloti* isolates as well as biochemical and genetic characterization under induced drought stress using PEG6000 [7].

RAPD –PCR was used to evaluate genetic variation between the isolates and select efficient strains to inoculate legumes and improve soil fertility in farming systems which has significant economical and ecological benefits.

Materials and Methods

Thirty isolates of *Sinorhizobium meliloti* were isolated from nodules of alfalfa (*Medicago sativa*) plants from different geographical sites in Iraq. After rinsing in 95% ethanol nodules were surface sterilized using 0.1% acidified mercuric chloride[8],squashed in 0.2 ml sterile water , then streaked into Mannitol Salt Yeast extract agar(MSY) plates .Single colonies of *S.meliloti* were isolated .The symbiotic ability of isolates was checked by plant infection test (PIT) [8]. All thirty isolates were tested for Gram stain , absorbance of congo red ,and changing the color of Bromothymol blue incorporated with MSY agar media .

Drought tolerance

The tolerance of isolates was tested by using polyethylene glycol -6000 (PEG-6000 w/v) in MSY broth media at different osmotic pressure ranging from -0.1 to -4 Mpa, (PEG-6000 did not used in the control treatment). Cultures incubated at 28˚C using a rotary shaker in the dark conditions for 7 days, then growing was assessed. Table (1) shows the geographical origin and nomination of *Sinorhizobium* isolates.

Table(1): Geographical and nomination of *Sinorhizobium* isolates .

Isolate no.	Geographical site	Isolate no.	Geographical site
Bs 2	Khanqeen 2 , Iraq	Bs 42	Kufa-zerga 7 , Iraq
Bs 57	Tarmea 2 , Iraq	Bs 43	Kufa-zerga 8 , Iraq
Bs 12	Amryea Fluja 2 , Iraq	Bs 44	Kufa-zerga 9 , Iraq
Bs 13	Seqlawea 1 , Iraq	Bs 46	Kufa –Abasea 2 , Iraq
Bs 15	Seqlawea 3 , Iraq	Bs 48	Nasrea , Msaib2 , Iraq
Bs 16	Seqlawea 4 , Iraq	Bs 49	Nasrea , Msaib3 , Iraq
Bs 23	Abu ghreb , Dr*. harb	Bs 50	Amryea Fluja 3 , Iraq
Bs 25	Babel , Kefil 2 , Iraq	Bs 53	Baquba 3 , Iraq
Bs 26	Babel , Kefil 3 , Iraq	Bs 54	Kufa –Qzwenea 4 , Iraq
Bs 29	Ramady 3 , Iraq	Bs 55	Mahawel 1 , Iraq
Bs 30	Ramady 4 , Iraq	Bs 57	Mahawel 3 , Iraq
Bs 31	Abu ghreb 1 , Iraq	Bs 58	Saydia-Baghdad , Iraq
Bs 32	Abu ghreb 2 , Iraq	Bs59	Ramadi 4 , Iraq
Bs 38	Nasryia , Iraq	Bs 60	Seqlawea 7 , Iraq
Bs 40	Balad 2 , Iraq		
Bs 41	Balad 3 , Iraq		

DNA extraction

Genomic DNA was extracted from eleven *Sinorhizobium* isolates by following the protocol of wizard genomic DNA purification kit(promeaga).The concentration and purity of DNA were estimated spectrophotometrically at 260 and 280 nm.

Fingerprinting of genomic DNA by RAPD-PCR: Six arbitrarily chosen primers used for RAPD fingerprinting were 10 nucleotides in length. primer sequences as follows:

5′ AAGGCGGCAG 3′ (OPI-06)
5′ CACACTCCAG 3′ (OPC-16)
5′ TCGTCGCAGA 3′ (OPJ-18)
5′ AAGCGACCTG 3′ (OPN-16)

5′ AGATCCCGCC 3′ (OPV-14)
5′ GTCCGTATGG 3′ (OPT-19)

All the primers used in this investigation were obtained from (Bioneer).The reaction mixtures (Bioneer master –mix PCR reaction kit) were incubated at 94 º C for 5 min for initial denaturation, 32 cycles in intervals of 1 min at 94 ºC, 1 min at 32 ºC and 1 min at 72ºC followed by 7 min of incubation at 72ºC in (multigen gradient. labnet) thermo cycler. PCR –amplified DNA fragments were separated by horizontal gel electrophoresis on 1.2% agarose gel. A molecular size marker, 1 kb ג Hind III digested DNA ladder (promeaga) was run in all gels. Electrophoresis was carried out at 5 v/cm and 25ºC during 3 h. The patterns were visualized using the gel documentation system.

DNA fingerprinting analysis: All RAPD fingerprintings and restriction patterns were converted into a two dimensional binary matrix (1. Presence of a band; 0, absence of a band) and analysed by using UPGMA algorithm to perform hierarchical cluster analysis and to construct a dendrogram.

Results and discussion

All the thirty isolates studied were rod-shaped bacteria on MSY media and grew at 28 C°. They were Gram^{-ve}, mucous and changed the color of Bromothymol blue +MSY medium into yellow.

Drought tolerance of isolates

Vast variation were recorded among the different *S. meliloti* isolates in relation to their drought tolerance. All isolates were able to grow on -0.1 , - 0.5 and -1.0 Mpa water potential plus the control treatment with 100 % growth percent, at - 1.5 Mpa water potential only nine isolates were able to grow at this level, while the isolate Bs58 only tolerated upto -1.0 Mpa water potential (drought sensitive). Twelve of the isolates showed good tolerant to low water potential ranging from -3.0 to-4.0 Mpa , they represent about 40% from total thirty isolates, it was drought tolerant. The last eight isolates tolerated upto-2.5 Mpa which it was drought moderate tolerant isolates as illustrated inTable (2) .

Table (2): *Sinorhizobium* isolates tolerant to drought stress

Drought Tolerant		Drought Sensitive		Drought moderate tolerant	
Isolate No.	Highest (MPa) tolerated	Isolate No.	Highest (MPa) tolerated	Isolate No.	Highest (MPa) tolerated
Bs 12	-4	Bs 15	-1.5	Bs 2	-2.5
Bs 23	-3	Bs 25	-1.5	Bs 7	-2.5
Bs 29	-3	Bs 26	-1.5	Bs 13	-2.5
Bs 30	-3.5	Bs 42	-1.5	Bs 16	-2.5
Bs 32	-4	Bs 43	-1.5	Bs 31	-2.5
Bs 38	-4	Bs 44	-1.5	Bs 46	-2.5
Bs 40	-3.5	Bs 54	-1.5	Bs 48	-2.5
Bs 41	-4	Bs 55	-1.5	Bs 49	-2.5
Bs 50	-3	Bs 57	-1.5		
Bs 53	-3	Bs 58	-1.0		
Bs 59	-3				
Bs 60	-3				

The decline in *Sinorhizobium meliloti* isolates growth with the increase of drought stress due to accumulation of salt, an increase in toxic compound which could reach toxical levels causing depression in viability also when water dips the RNA-polymerase ceases to function and metabolism stalls [9]. Some researches [2] reported that in osmotic stress a specific protein formed which was detected as new protein band in dodecyl sulfate poly-acrylamide gel electrophoresis. Rhizobia accumulate osmolyte in response to the osmotic stress which helps them to overcome effect of osmotic stress[10].

DNA fingerprinting by RAPD –PCR

Total genomic DNA from all isolates were used as templates in amplification reaction with RAPD sets of primers. The PCR products from different isolates were separated by horizontal gel electrophoresis on 1.2 % agarose gel.

The primers yielded multiple DNA products ranging in size from 2500 to 150 base pair (bp). The number of amplified fragments ranged from 1-8 per isolate, depending on the primer used in the amplification reaction as represented in Figure (1).

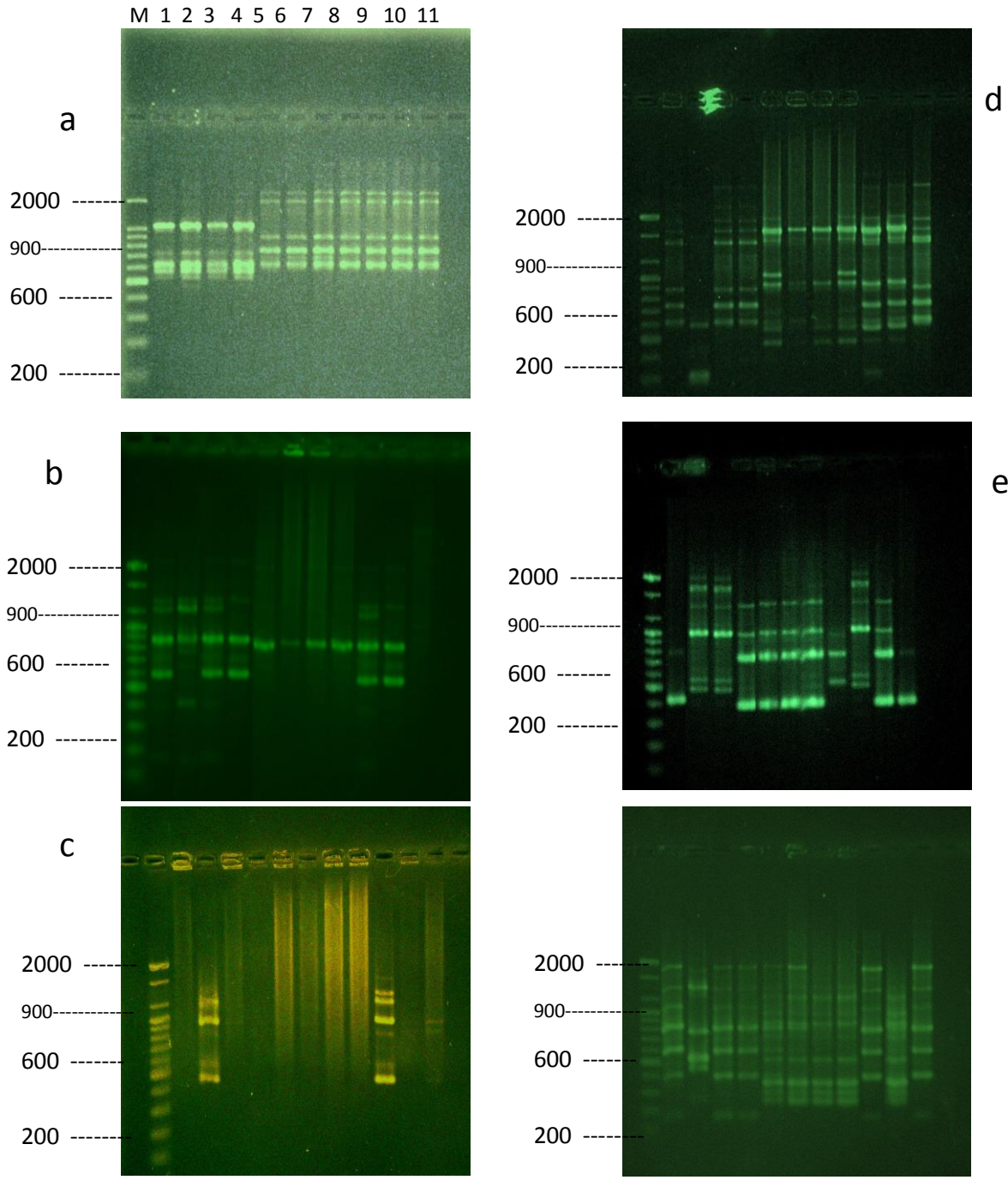

Fig (1) RAPD fingerprints of Sinorhizobium isolates generated by primers OPJ - 18 (a) , OPC -16(b) , OPT-19(c) , OPI-06(d) , OPV14(e) and OPN- 16(f). Lane M-1kb DNA marker ; 1-Bs 12 , 2 -Bs30 , 3-Bs38 ; 4-Bs41 ; 5-Bs44 ; 6-Bs 54 , 7- Bs 55 ; 8-Bs58 ; 9-Bs 49 ; 10-Bs 31and 11-Bs 16.

The dendrogram obtained by numerical analysis (past program v 1.92) of gel data. Figure (2) shows that all tested isolates could be divided into two major cluster (similarity level of 100) only three *S. meliloti* isolates were grouped within the first major cluster all three were drought and salt tolerant isolates, also isolate Bs 12 tolerated high temperature 42ºC (data under publishing). The second major cluster comprised most of *S. meliloti* isolates (8 isolates) and it subdivided into two subcultures with similarities of 53%. The first subcluster included Bs 49 and Bs 16 which were moderate drought tolerant isolates the similarities between them were 68%, the second subcluster divided into two subgroups, first one included Bs 30 which was a drought tolerant isolate, and the second subgroup included all four drought sensitive isolates plus Bs 31 which was moderate drought isolate. The dendrogram shows 90.5 % similarity level between the two drought sensitive isolates Bs 44 and Bs

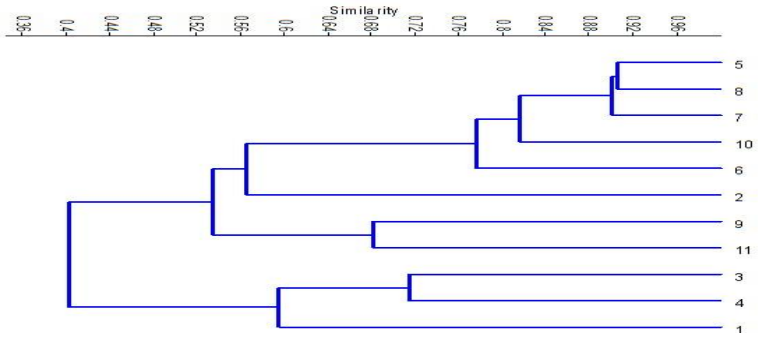

Fig. (2): UPGMA dendrogram showing the similarities values among eleven *Sinorhizobium* isolates on the basis of RAPD –DNA fingerprinting ,1 - Bs 12 ;2- Bs 30 ; 3- Bs 38 ; 4- Bs 41 ;5- Bs 44 ; 6- Bs 54 ; 7- Bs 55 ;8- Bs 49 ; 10- Bs 31 and 11 – Bs16.

In the next few years grain legumes would be used as means of Nitrogen investment in cropping systems in response to the reduction of non-renewable source of energy. Moreover grain legume crops are largely influenced by water stress. While the world area exposed to drought periods may increase in the coming years as a result of global warming.

PCR based techniques are faster and generally superior to more classical cell typing methods. It has been frequently used for identification and differentiation of bacterial isolates such as *Rhizobium* [11, 12,13] reported that RAPD-PCR is suitable for the fingerprinting of *Sinorhizobium meliloti* isolates and differtiating between closely related strains.

Likewise Gisele *et al.* (1996) [14] indicate that RAPD –DNA fingerprinting provide an efficient means for rapidly typing a large number of strains under well standardized experimental conditions.

According to Noshin *et al.* (2008) [15], RAPD is a potential tool for the identification of the genetic and systematic of different populations.

Possible reason for genetic diversity might be that low soil moisture may have resulted in genetic adaptations of the strains. However variation among different strains of heterogeneous origin suggested that there is genetic potential to drought stress. Its well documented that Rhizobia are capable of surviving under low water potential.

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