Morphological, Biochemical and Molecular Characterization of Ten Rhizobial Bacteria Isolates.

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Abstract

This study was done in biotechnology laboratories in the national center of organic farming /ministry of agriculture where ten of Rhizobial isolates and strain studied were either local isolate from chickpea root nodules or non- local (Syrian and Turkish) obtained from ICARDA.These isolates were identified and characterized on the basis of colonies morphology and biochemical tests including gram staining, catalase and oxidase tests. The Genetic diversity among the isolates was assessed by RAPD(Randum Amplified Polynorphic DNA)-PCR(Polynerase Chain Reaction) finger printing by using five primers. The RAPD result showed high ability to detect genetic polymorphism in Rhizobia and have the ability to generated unique bands (marker) especially in Shiekhan 3(10)bands Mosle(8)bands isolates that isolated from chickpea plants .

Key word: *Rhizobium*, molecular,RAPD-PCR,Biochemical,

دراسة الصفات المظهرية والكيموحياتية و الوراثية لعشر عزلات من بكتريا الرايزوبيا

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الخلاصة

تمت الدراسة الحالية في مختبرات التقانات الإحيائية في المركز الوطني للزراعة العضوية التابع لوزارة الزراعة حيث تضمنت دراسة عشر عزلات و سلالات من بكتريا الرايزوبيا ، تضمنت عزلات محلية عزلت من العقد الجذرية لنبات الحمص والباقلاء أو غير محلية ذات مصدر سوري أو تركي تم الحصول عليها من منظمه ايكاردا. شخصت العزلات بالاعتماد على المظهر الخارجي والفحوصات الكيموحياتيه عن طريق صبغة كرام و فحصي الأوكسيديز و

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الكاتاليز .درست التغايرات الوراثية للبكتريا على اساس تقنيه ال RAPD–PCR باستخدام خمسه بادئات حيث اظهرت هذه التقنية قدره عاليه للتحري عن التباين الوراثي في الرايزوبيا وقدره البادئات على إظهار حزم مميزه للعزلة وكانت أكثر العزلات التي ظهرت فيها هذه الحزم هي شيخان 3 (10)حزم وموصل (8) حزم والتي عزلت من نباتات الحمص.

Introduction

The fast – growing *Rhizobium* spp. and the slow growing *Bradyrhizobium spp.* or root nodule bacteria are medium – sized, rod- shaped. Cells 0.5-0.9 µm in width and 1.2-3.0 µm in length. They do not form endospores, gram negative, and are mobile by single polar flagellum or two, six peritichous flagella. Optimal growth of most rhizobial strains occurs at temperature range of 25-30^{o}C and ph 6-7[1].

Rhizobia form intimate symbiotic relationships with legumes by responding chemotactically to flavonoid molecules released as singles by the legume host. These plant compounds induce the expression of nodulation node genes in rhizobia, which in turn produce lipo – chito – oligosaccharide (lco) singles that hrigger mitotic cell division in root leading to nodule formation [2]. A number of reports have demonstrated the ability of rhizobia to colonise roots of non – nitrogen fixing plant and localize themselves internally in tissue including the xylem [3].Early work revealed that *Rhizobium* spp. Have the potential to produce extra cellular compounds with direct antimicrobial activities [4]. Indicating that antibiosis maybe part of their reported biocontrol efficacy. Most researches results indicate that *Rhizobium* inoculation is promising fertilizer because it is cheap , easy to handle and improves plant growth [5,6].Genetic variation of rhizobia such as *Mesorhizobium ciceri* has been studied, in the use of strain typing [7]. Antibiotic resistance[8].

And the ability of bacteria to utilize different component for carbon source [9] .But all of these methods did not identify the realy genetic variation

The molecular analysis consider the most useful technique to determine the genetic variation between the isolate and strain of bacteria [10] . One of the most important technique is (PCR). polymerase chain reaction [11], which was the essential reason for development a lot of DNA markers and make a revolution in the biotechnology, and one of these DNA marker is random amplified of polymorphic DNA (RAPD) which was discovered in the 1990 and it was the first technique which use widely because it is easily and non-cost, rapidity and précisionly [12]. So it used and depended on the recognizing of strain of *Rhizobium* [13] . The present study reports the isolation of Rhizobial isolates from chickpea and fababean plants and their characterization on the basis of molecular ,bio chemical and morphological characters.

Material and methods

1. Sample collection

In this study 8 local isolates of *Rhizobium* spp. were used and two were non local. Six isolates were isolated from chickpea root nodules. And the other isolates were obtained from fababean root nodules. According to [14]. The non local isolates were strains obtained from ICARDA. The local isolates were coded according to the region where the plant taken from. and given the number as below table 1.

Table.1- Name and number of Rhizobial isolates

No. isolates	name	Scientific name	origin
1	Mosle	*Mesorhizobium Ciceri*	local
2	Erbil	*Mesorhizobium Ciceri*	local
3	Sheikhan 3	*Mesorhizobium Ciceri*	local
4	Tarmia	*Rhizobia leguminosarum*	local
5	Rhashdia	*Rhizobia leguminosarum*	local
6	Duhok 1	*M. Ciceri*	local
7	Aqra	*M. Ciceri*	local
8	Alqoosh	*M. Ciceri*	local
9	CP-20	*M. Ciceri*	ICARDA Syrian
10	CP-93	*M. Ciceri*	ICARDA Turkish

The media of *Rhizobia* which described was mannitol yeast extract agar (MYA)

Identification of bacteria

After 48-72 h from culturing at 28^{o} C bacteria on the MYA media the color and shape of colonies was observed under light microscope. Gram staining was done by the method of [16].Oxidase test was performed to determine the presence of oxidase enzyme in bacterial isolates [17]. Kovac reagent (1%N, N,N.N-tetramethyle-p-phenylene diamine) was dissolved in warm water and stored in dark bottle. A strip of filter paper was dipped in this reagent and air-dried with the help of sterile wire loop, one-day-old rhizobial colonies from agar plates were transferred on this filter paper strip.

The oxidase positive colonies turned lavender colored which became dark purple to black in color within 5 min.

Catalse test was preformed to study the presence of catalase enzyme in bacterial colonies. Rhizobial colonies (24 h old) were taken on glass slides and one drop of H2O2 (30%) was added . A presence of gas bubble indicated the presence of catalase enzyme [18].

DNA extraction

DNA was extracted from Rhizobial isolates according to the method of this method called salting out. [19]. Culture from each Rhizobium isolates were streaked on MYA media in plates and incubate at 28C for 2-4 days. Harvesting of bacteria done by added 2.5ml from SET solution (NaCl 75mM+ EDTA 25mM+Tris – HCl 20 mM ph 8), then transferred by micropipette to test tube. 250ml from SDS 10% are added then shacked very well after that it was transferred in a shaking water bath at 55^{o}C for two hours. After finish of this stage, 1ml from NaCl 5M solution was added and shacked very well. Left in the room temperature until the temperature of them become 37^{o}C then 2.5ml from chloroform was added and shacked by hand for 30 min. centrifugation for 15 min at 6000 rpm done, then take the supernated to another clean test tube, isopropanol was added at 1/6 volume of the mixture and shacked very well for 3 min. the DNA appear like a filiform – DNA was drawn by Pasteur pipette seild from the end by heating and crooked end to be like a hook. The DNA was washed by ethanol 70% and dried by air then re-dissolved by 50 ml sterile D.W. [20] .The concentration and putity of DNA was estimated spectro-photometrically at 260-280 nm.Sample was mixed with loading buffer (2 µl sample+3 µl loading) and loaded into the well on agarose gel 1.00%. then gel were run horizontally in 1x TBE buffer. DNA bands was visualized by U.V.at 360 nm wave length. A gel documentation was used to document the observed bands.

RAPD_PCR analysis of genomic DNA

Genetic diversity and polymorphism among isolates was analyzed by RAPD_PCR technique adapted by [21]. For RAPD_PCR 10 oligonucle-otide (5) primer were used.

The primers were:

OPA-10	GTGATCGCAG
OPA-11	CAATCGCCGT
OPA-12	TCGGCGATAG
OPC-16	CACACTCCAG
OPN-16	AAGCGACCTG

In RAPD_PCR technique master mixture provided by Bioneet\Korea RAPD final reaction volume 20 ml contained 5 ml of(1x) master mix,2 ml of 10 pmol primer,2 ml of 50 ngm\ml genomic DNA and the rest of the volume was adjusted with sterile pure water.

PCR program was according to [22] when the initial denaturation step 94^{o}C for 4 min, denaturation step 94^{o}C for 30 sec. annealing 35^{o}C for 30 sec, and the extension 72^{o}C for 1 min. these three steps were repeated for 35 cycle, the final extension step was at 72^{o}C for 10 min. Amplified PCR products were separated by gel electrophoresis on 1.5% (w/v) agarose gel and visualized under U.V. gel documentation after staining with ethedium bromide (0.01g/ml).1kb DNA ladder (Bioneer) was used as molecular marker**.**

Analysis of RAPD product

The band that produced from the amplification has been calculated from the image of the electrophoresis the agarose since the signal (+) mean found of the band, and (-) mean the absence of the band with calculate molecular weight.

The effecincy of primer estimated by using

e.of primer = total number of primer bands *100
total number of all primer bands

the discriminatory power %

= n.of polymorphism band of the primer*100
n.of polymorphism band to all primer[18,19]

Result and discussion:

All isolates that isolated from chickpea and fabapean were found to have circular colonies with regular borders,creamy in color ,showing intermediate to high, production of mucus on MYE media .all isolates were positive for catalase and oxidase test as table 2

Table.2- Morphology and biochemical test of Rhizobial isolates

isolates	Gram stain	Colony morphology	Catalase test	Osidase test
Mosle	-	Circular	+	+
Erbil	-	Circular	+	+
Sheikhan 3	-	Circular	+	+
Tarmia	-	Circular	+	+
Rashdia	-	Circular	+	+
Douhok 1	-	Circular	+	+
Aqra	-	Circular	+	+
Elqoosh	-	Circular	+	+
CP_20	-	Circular	+	+
CP_93	-	Circular	+	+

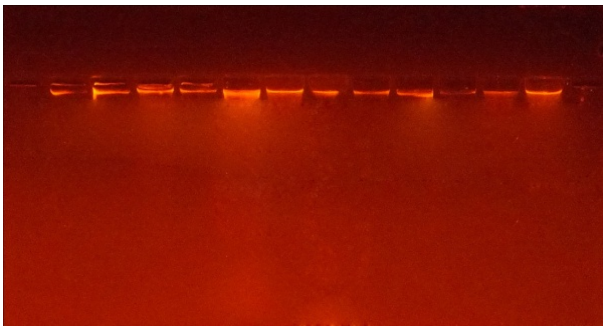

Figure.1- Genomic DNA of Rhizobial isolates(1. Mosil 2. Erbil 3. Shiekhan3 4. Tarmia 5. Rashidia 6.Duhok1 7. Aqra 8. Alqoosh 9. Cp-20 10. Cp-93) electro photesis on 1.0% agarose gel

RAPD_PCR analysis

The genomic DNA of rhizobia yield about 11mg/ml of bacterial growth the purity of the DNA ranged between 1.4-1.7 figure 1. Primers used in this study reacted with bacterial genome and gave results in term of amplification and polymorphism, these primer were recognized to the sequence of nucleotide complementary to the template DNA and repeat the RAPD_PCR for each primer.

Each sequence on the genomic DNA complementary to any primer lead to appearance of band on the agarose gel in result number of the resulting bands refer to the number of sequences on the genomic DNA that complementary to the primer and the primer recognized it for amplification. Absence of the any band in the

isolate or species or any other sample mean absence the site of primer in this genome [23].

1. A-10 primer

This primer showed in Rhizobial isolated DNA number of bands in range 3-6 bands with molecular weight 0.1-2.5 Kb, like the fourth band with 1.3 kb which appeared in Sheikhan 3 isolate and band number five which appeared in Mosle isolate had 1.2 kb and bands number six appeared in all isolates, with molecular weight 0.8 Kb. and the twelfth band with 0.1Kb. There is also band number seven with 0.7Kb., eleventh band 0.4Kb., eight 0.65Kb. These in the additional to the fourth, fifth, twelfth band, considered unique bands. So in this primer there is six unique bands.

2. A-11 primer

The use of primer A-11 in PCR technique to amplify genomic DNA of Rhizobial isolates produced number of bands ranged 4-7 band with molecular weight 0.2-2.1kb like the eighth band with 0.8kb molecular weight which appeared in Alqoosh isolate only and the fifth band which absent in Shikhan 3 isolate only and the sixteenth band with 0.2kb which appears in Mosle isolate only. And fifteenth band with 0.25 kb that's appears in CP-93 only. This primer generated four unique bands these were eighth, twelfth, fifteenth and sixteenth.

3. A-12 primer

In PCR reaction of primer A-12 the result appeared 2-6 bands on the agarose gel with molecular weight 2.8 -0.1bp for example the band number 1 with 2.8bp and the band number four with 2.1bp that appeared in Mosle isolate only and the band number ten with 0.75 bp these appeared in Rashdia and CP-93 isolates , and the band number fourteen that appeared in shiekhan3 only,.see tables (4- 10). This primer distinguished seven unique bands which are band number one, two, four and five in Mosle isolate, band number three, eleven and fourteen in shiekhan 3 isolate.

4. C-16 primer:

This primer produced on agarose gel bands range 3-8 with molecular weight 1.4-0.2 kb like the third band with 0.1 kb which absents in Shikhan 3 isolate only and sixth band with 0.8kb which appeared in the Shiekhan isolate only and the band number eleven that appeared in Erbil only.

In this primer two unique band resulted that sixth band and eleven

5. N-16 primer

N-16 primer reacted with total genomic DNA and produced 6-10 bands with molecular weight 2.8-0.1kb example for these bands the second bands with 2.7kb these appeared in Shiekhan and Tarmia and absent in other isolates and the tenth band with 1.4 kb which appeared in Shiekhan isolate only and eleventh band that appeared in Rhashdia isolate only and the two bands number fifteen these absents in Alqoosh isolate and CP-93 with 0.7kb and band number twenty with 0.2kb these appeared in Alqoosh and CP-93 only and the final band with 0.1 kb that appeared in all the isolates). So N-16 primer generated three unique bands, two of three were in Shiekhan 3 isolate tenth and thirteenth band 1.4,1.0 Kb in molecular weight and band number eleven in Rashdia with 1.3 Kb.

Figure 2 shows the results of all primers.

Table3-The efficiency and distinction of bands that resulting from the primers**.**

The primers	The efficiency of the primer %	Discriminatory power %
OPA-10	10	8.9
OPA-11	11.6	13.8
A-12	10	11.3
C-16	13.3	9.7
N-16	16.6	16.2

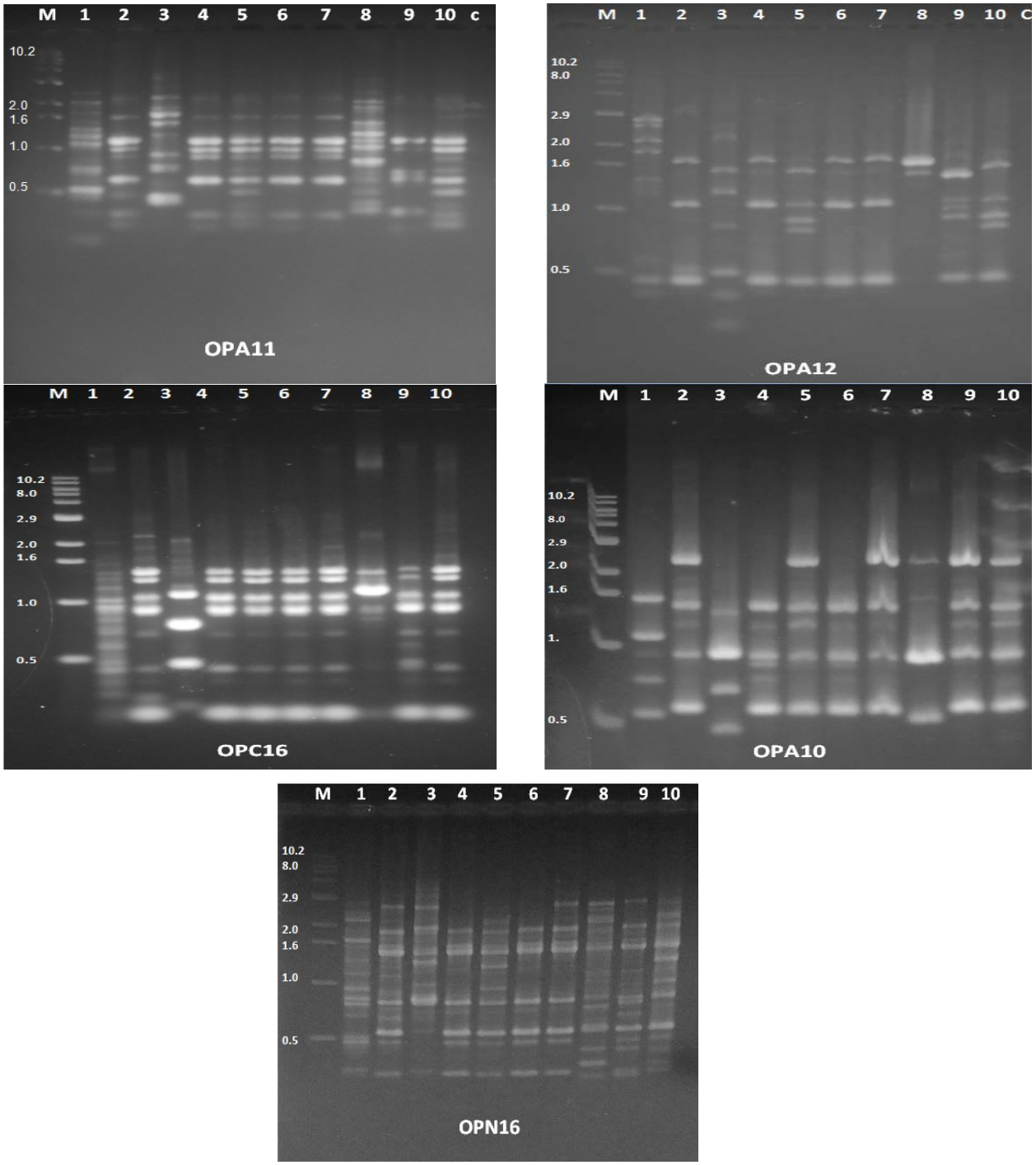

Figure 2- Result of amplification of OPN-16 ,OPA-10 ,OPA-11, OPA-12,OPC-16 on the 1.2 agarose gel with 100 bp DNA ladder ; (1- Mosel, 2- Erbil, 3- Shiekhan3, 4-Rashdia, 5- Tarmia, 6- Duhok1, 7-Aqra, 8-Alqoosh, 9- Cp-20, 10- Cp-93)

detect genetic polymorphism in rhizobia such as N-16 and A-11 primers. RAPD techniques have the ability to generated unique bands (marker) for the rhizobial isolate using in present study. Most of the unique bands appeared in (the Shiekhan 3, and Mosle) isolates like C-16 primer which reviled aunique bands profile with this isolate as table 4.

Table.4- The unique bands appears from primers

Primr-es	Name of isolate	Molecu-lar weight kb	Type of band
A-10	Shiekhan3 Mosle Mosle Skiekhan3 Shiekhan3 mosle	1.3 1.2 0.7 0.65 0.4 0.1	marker
A-11	Shiekhana3 Alqoosh Shiekhan3 Cp-93 mosle	2.1 0.8 0.4 0.25 0.2	marker
A-12	Mosle Mosle Shiekhan Mosle Mosle Shiekhan shiekhan	2.8 2.7 2.3 2.1 1.9 0.5 0.1	Marker
C-16	Shiekhan Erbil	0.8 0.3	Marker
N-16	Shiekhan Rashdia shiekhan	1.4 1.3 1.0	Marker

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