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Article

Detection of *Rhizobium leguminosarum* bv. *trifolii and Rhizobium leguminosarum* bv. *phaseoli* isolated from root nodules of leguminous plants by multiplex-PCR

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ABSTRACT

The genetic diversity of 4 R. leguminosarum bv. trifolii and R. leguminosarum bv. Phaseoli that was extracted from root nodes of Trifolium spp and Phaseolus vulgaris L cultivars circulated in Mosul soils were investigated. The bacteria strains under study show similar tolerance levels against some external factors. In the current study, using classical diagnosis methods, similarity and distance indices and DNA polymorphism were examined with the randomly amplified polymorphic DNA. The isolates were distributed through genetic diversification into two main classes, including two main branches, Rl2 and the other sub-group was divided into Rl3, and the neighboring group involved Rl1 and Rl4. It is noted that individual plants and abiotic aspects were less affected than the Genetic factor on bacterial diversity. On the other hand, in multiplex-PCR reaction, Our outcomes revealed specific amplified for R. leguminosarum bv. trifolii with amplification products 419 bp and R. leguminosarum bv. Phaseoli with amplification products 362 bp.

Keywords: R. leguminosarum bv. phaseoli, R. leguminosarum bv. trifolii , nodules, multiplex-PCR, RAPD-PCR .

INTRODUCTION

Rhizobia belongs to legumes that are widespread all over the world in various kinds of soil. They can be found as symbionts of leguminous plants. Rhizobia can stabilize the amount of nitrogen to a sufficient level to lessen the plants' dependence on nitrogen artificial fertilizers [1]. The host bezel of R. leguminosarum is capable of stimulating successful symbiosis with different legumes that belong to the family of Trifolium, Phaseolus, Vicia, Lathyrus, Pisum, and Lens that form several biovars (symbiovars) and species [2]. Trifolium (clover), An important leguminous plant, Can utilize benefit nitrogen (N2) and install sufficient quantities of it in the soil during growth [3]. Like other members of Phaseolus vulgaris be-

longing to Leguminoceae, it established a useful symbiotic conglomeration with Rhizobium leguminosarum bv. Phaseoli that lead to form root nodules and fixing nitrogen [2]. Rhizobium leguminosarum bv. phaseoli and Rhizobium leguminosarum bv. trifolii are gram-negative bacteria that can interact with the roots of Trifolium spp. and Phaseolus, respectively, to shape active nitrogen-fixing nodules [4, 2]. Abiotic factors like water stress, soil salinity, pH, temperature variation and Soil nature affected the activity of nodules formed [5]. A few studies are present about rhizobial nodules that deal with Trifolium (clover) and Phaseolus vulgaris roots. Therefore, it is necessary to investigate those isolates' genotypic and phenotypic diversity. The present study focuses on the isolation of Rhizobium leguminosarum bv. trifolii and Rhizobium leguminosarum bv. Phaseoli in Mosul city from legume plants. Thus, the current study aims to classically demonstrate the strains depending on the isolation sites, different biochemical tests and ability to grow in different Concentrations of NaCl and dried environment conditions. In contrast, the molecular screening achieved sensitive PCR protocol for the specific PCR by the23S rRNA partial gene [6], which is very useful for detecting isolated strains [7]. It is worth mentioning that random amplified polymorphic DNA (RAPD) has been utilized to evaluate the rhizobia diversity to enhance the phylogenetic tree and relationships between bacteria.

MATERIALS AND METHODS

Bacterial strains

Four isolates of Rhizobium leguminosarum bv. trifolii and R. leguminosarum bv. Phaseoli were recovered from active nodules of Trifolium spp (Two isolates)and Phaseolus vulgaris L(Two isolates) root plants. All nodules were washed with tap water; after being impregnated in 95% ethanol, they were washed with distilled water and cultivated on yeast extract of mannitol agar medium. So single cell colonies of R. leguminosarum bv. phaseoli and R. leguminosarum bv. trifolii were prepared in pure form [8].

Temperature Adaptation and NaCl Tolerance

The isolated bacteria were tested for various temperatures, beginning at 28,33,42 and ending at 53 ^{o} C onto YEM. Salinity tolerance of isolated strains was conducted using YEMA agar containing 2.5,5.0,7.5 and 10% NaCl. Normal saline 8.9%NaCl was added to the YEMA medium as a standard test [8].

Primary diagnosis and biochemical reaction

Pre-diagnosis for bacterial isolates includes macroscopic examination and biochemical tests from active nodules of Trifolium spp. and P. vulgaris plants [9].

Isolate and evaluate of Genomic DNA

Genomic DNA was extracted using a gDNA purification kit (Promega, USA). Its purity was conducted by nano-spectrophotometer at 260-280nm. gDNA integrity was estimated by electrophoresis [10].

Molecular detection of R. leguminosarum bv.

For investigation of Rhizobium leguminosarum bv. trifolii and R. leguminosarum bv. Phaseoli, respectively, two primer sets were used in this field provided by (Bioneer, Korea), as detailed in the following table(1).

Gene		Sequence (5'- 3').	Amplicon size(bp.).	Accession No.(NCBI).
16S-IS-23SrRNA*	F	TTT-GCTGTCAGGTGTTTGCG	419	GQ411394.1
	R	AACGG-TAATCCGATCGTCCG		
23SrRNA**	F	AGCGTTCCGTAA-GCTGATGA	362	U47356.1
	R	GTTCTCTCAA-GCGCCTTGGT		

Table 1. Primer sets for molecular screening of R.leguminosarum bv. ****R. leguminosarum bv. trifolii* **, ** *R. leguminosarum bv. Phaseoli***.**

Multiplex reaction was performed program, which was begun with an initial denaturation at 95°C for 10min, 35 cycles of 95°C for 30s,54°C for 30s,72°C for 30s and 5 with final extension at 72°C utilizing AccuPower® Multiplex PCR Pre-Mix(Bioneer, Korea).

2.6 Genetic variation of gDNA according to RAPD-PCR

Four randomly chosen primers (Bioneer, Korea), utilized for RAPD fingerprints range composed of ten nucleotides and their sequences and symbols include: OPA-18(AGGTGACCGT), OPA-16(AGCCAGCGA A), OPH-01(GGTCGGAGAA), OPK-01(CATTCGAGCC). The previous primers were synthesized by Bioneer (S. Korea). PCR primer (20µl.) included Taq DNA polymerase 1U,dNTPs 250µM.(dATP, dCTP, dGTP,dTTP), 1.5 mM of MgCl, 30 mM of KCl, 10 mM of Tris-HCl(pH 9.0), methylene blue as a loading dye, nuclease-free water and DNA marker was 100bp. (Bioneer, Korea). The amplification tubes were subjected to Applied Biosystem gradient thermocycler (USA) for achieve following program: initial incubation 95^{o} C for 180s, and 35cycles of 95^{o} C for 30s as initial denaturation, 33,35,30 and 30^{o} C (Respectively)for 30s as annealing temperature for primer hybridization,72^{o} C for 30s as an extension and final incubation 72^{o} C for 180s.

2.7 DNA fingerprinting analysis

Data analysis of 16S-IS-23SrRNAgene for detection of R. leguminosarum bv. trifolii and 23SrRNAgene for detection R. leguminosarum bv. Phaseoli using MEGA version 6 [11].

All RAPD outcomes were transformed into a binary matrix with two digits (0, absence of a band and 1, presence of a band)to achieve genetic diversity and phylogenetics utilizing the PAleontological software of Statistics (Version 1.94b; Software of PASS, University of Oslo).

RESULTS

Samples

Four isolates were collected, two from Trifolium spp root nodules and the others from Phaseolus vulgaris root nodules Table (2).

Plant	isolated strains	symbol
Trifolium aureum L.	1	Rl1
Trifolium repens L.	1	Rl2
Phaseolus vulgar-is(Locally)	2	Rl3, Rl4
Total isolated strains	4	

Table 2. Source of Rhizobium spp.

Phenotypic investigation

The similarity among all isolated strains was dominant depending on phenotypic determination, as detailed in Table 3 as found by (Deka and Azad, 2006; Gilbert et al.,2018),

Test			Bacterial strains			
			R11	R12	R13	R14
Direct exami-nation	Microscopically		Gram-negative rod			
Indirect exam-ination	Cultural appearance		Creamy mucoid			
Biochemical test	Oxidase					
	Catalase		Positive			
	Glucose and scrose fre-mentation		Positive			
	I		Positive			
	O		Negative			
	Vi		Positive			
	Ci		Negative			
	Ca		Negative			
	3-K		Positive			
Tolerance	Salinity (2.0-10%)		Resist (Growth rate reduction)			
	Dreid Enviorment		Resist			

Table (3): Microscopic, macroscopic, biochemical and tolerance of Rhizobium spp.

Significance of 16S-IS-23SrRNA and 23SrRNA

Our outcomes revealed specific amplification for DNA of R. leguminosarum bv. trifolii with amplification products 419 bp and R. leguminosarum bv. Phaseoli with amplification products 362 bp. as shown in Figure 1.

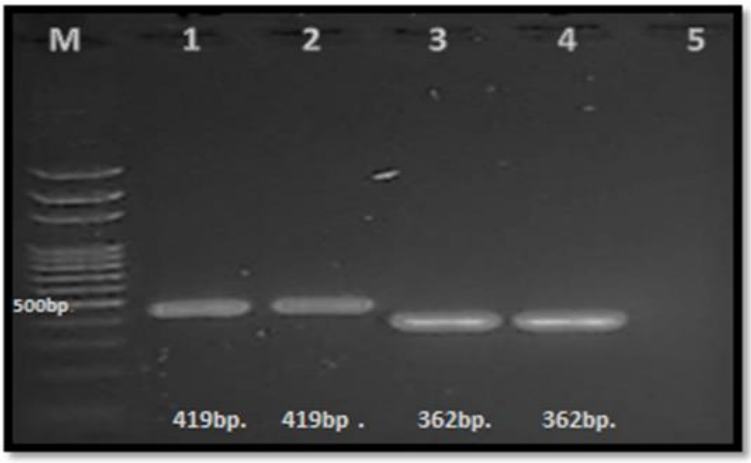

Figure 1 . Amplicons of for *R. leguminosarum bv. trifolii* (Lines1 and2, 419bp.)and *R. leguminosarum bv. Phaseoli* (Lines 3 and 4 ,362bp). Line M is a DNA marker (100bp.) and line 5 is a negative control.

The notable amplification fragments of OPA-18 (AGGTGACCGT), OPA-16 (AGCCAGCGAA),OPH-01 (GGTCGGAGAA), OPK-01 (CATTCGAGCC) explored more accurate relationships among Rhizobial isolated strains (Figure two and three) which in turn was represented via similarity and dissimilarity and dendrogram.

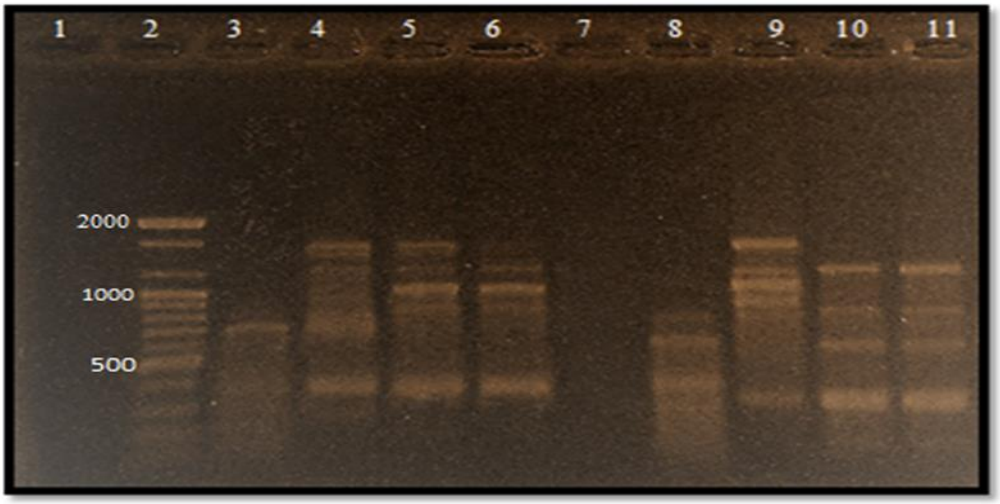

Figure (2). Random amplicons of R. leguminosarum bv. Trifolii (Lines 3-6) and R . leguminosarum bv phaseoli (Lines 8-11) primers. Lines 1 and 7 are negative control. Line 2 is a DNA marker (100bp.).

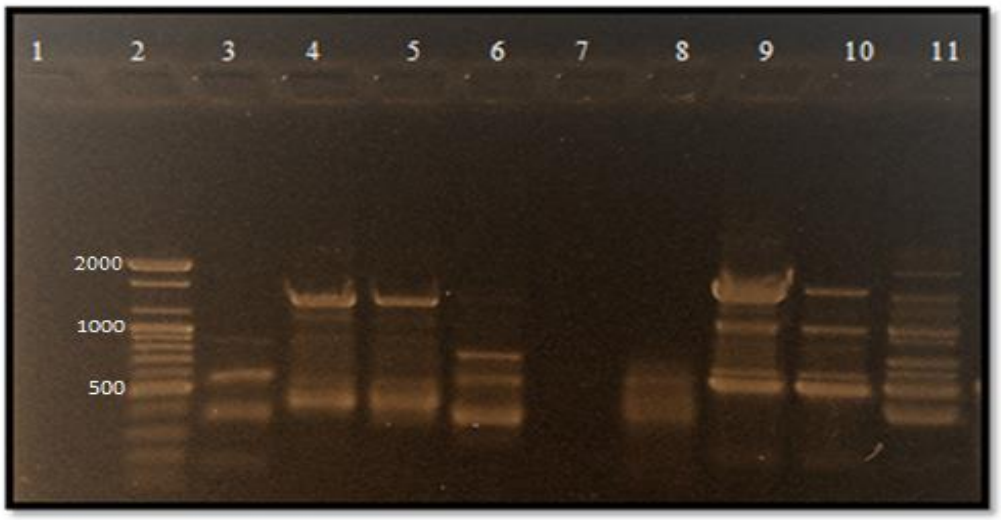

Figure (2). Random amplicons of R. leguminosarum bv. Trifolii (Lines 3-6) and R . leguminosarum bv phaseoli (Lines 8-11) primers. Lines 1 and 7 are negative control. Line 2 is a DNA marker (100bp.).

Similarity and distance indices

The observative data that belong to isolated strains revealed a high percent between Rl1 and Rl4 (0.62554) and a low between Rl2 and Rl3 (0.32969); more details in Table 4.

Symbols of rhizobium	Rl1	Rl2	Rl3	Rl4
Rl1	0	0.53161	0.51075	0.62554
Rl2	0.53161	0	0.32969	0.57104
Rl3	0.51075	0.32969	0	0.51075
Rl4	0.62554	0.57104	0.51075	0

Table (4): Similarity and distance indices among R. leguminosarum bv. trifoli and R. leguminosarum bv. Phaseoli isolates.

On the other hand, the illustrated dendrogram classified the isolated strains into clusters, including two main branches, Rl2. The other sub-group was divided into Rl3, and the neighboring group involved Rl1 and Rl4 (Figur 4).

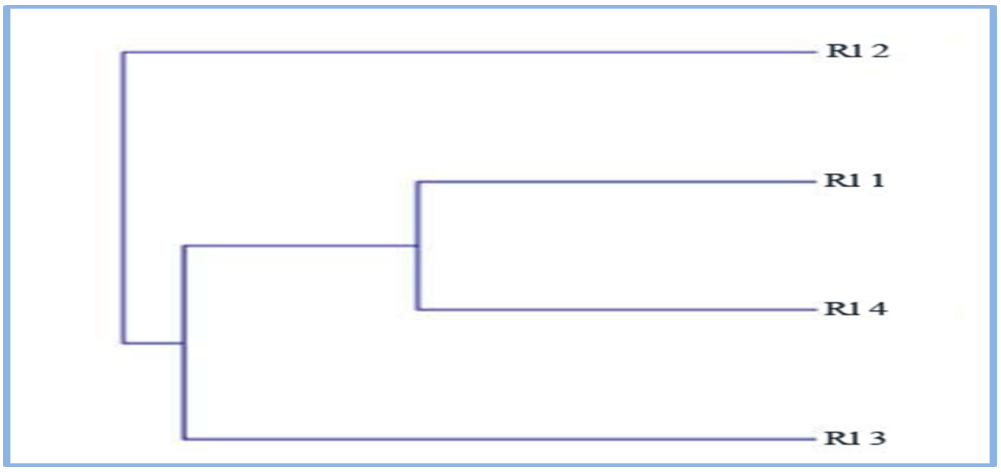

Figure (4) : Dendogram of R. leguminosarum bv. trifoli and R. leguminosarum bv. Phaseoli isolates revealed two main groups, one represented by Rl2. The subgroup was divided into two branches involving Rl3 and Rl1, and Rl4 represented the neighboring subgroup.

DISCUSSION

As for *Phenotypic investigation, p*revious results matched those documented by [12], who isolated Rhizobium from Phaseolus vulgaris L. Rhizobial isolated showed notable ability to various salt concentrations (2.0-10). Our conclusions and results were consistent with that reported by [13]. In addition, the results show growth reduction with increasing soil salinity and other non-suitable environmental conditions [14].

These results were concluded by [15,16,] and 17, who confirmed their outcomes by molecular determination utilizing specific primers for Rhizobial fingerprinting and genotyping. Generally, genotyping depending on specific primers allows the detection of bacterial genera and species [18].

RAPD is an important confirmative tool at the molecular level due to utilizing the same random primers with numerous species or genera [19].

Finally, similarity and distance indices results were agreed with reported data by [20].

Overall, we conclude that RAPD fingerprinting is a great tool that could be used to determine the variation among the Rhizobium population; many studies were carried out in this field, like (7; 21) who noticed that RAPD-PCR was more sensitive to demonstrate the relationship among rhizobium.

CONCLUSIONS

The phenotypic allows us to sensitivity at the genus level, As it was shown that all the isolates belong to the rhizobium, While the PCR multiplex reaction allows us to sensitivity at the species level As it classified bacteria into two groups, the R. leguminosarum bv. trifoli and R. leguminosarum bv. Phaseoli isolates. In contrast, rapid amplification led to the identification of Similarity and distance indices between all isolates.

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