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Molecular Identification of *Rhizobium* Isolates Nodulating Faba Bean Plants in Egyptian Soils

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Abstract

Eleven isolates of *Rhizobium leguminosarum symbiovar. Viciae* were isolated from root nodules of *Vicia faba* L. cultivated in 11 fields and represent different governorates in Egypt. The genetic diversity among the isolates was studied using the 16S rRNA gene partial sequence. The phylogenetic analysis formed two groups of isolates and the values of genetic distances were variable among the studied isolates. The highest value of genetic distance was between the isolates RL6 of North Sinai and RL8 of Dakhalia, while the lowest value was between isolates RL9 of Giza and RL10 of Sharkia. The isolates were evaluated for their tolerance to heavy metals using concentrations of 0.5, 1 and 2 mM of heavy metals (Cu, Pb and Zn). The ability to resist the heavy metals decreased with increase in concentration. At the highest concentration (2 mM), No growth was obtained with addition of Zn and Mn to the growth media, however only 27% of isolates could survive with the same concentration of Pb.

Keywords: Rhizobium; Legumes; Heavy metals

Introduction

The Legumes are the third largest family of higher plants with more than 650 genera, 18.000 species and are second in agricultural importance [1]. Legumes are grown on approximately 250 Mha and able to fix about 90 Tg of N_2 per year as result of symbiosis with *Rhizobia* [2]. *Rhizobia* are diverse group of eleven genera of Gram-negative unicellular soil bacteria which are able to induce nitrogen-fixing nodules on the roots of leguminous plants as *Vicia faba* L. The symbiosis between *Rhizobium* and legumes species is very important and accounts for 50% of 175 million tons of total biological nitrogen fixation used in agriculture. The *Rhizobium leguminosarum symbiovar. Viciae* is among fast-growing rhizobia and able to nodulate *Vicia sativa* and *Pisum sativum* [3].

Many papers focused on the genetic diversity of *Rhizobium* isolated from several countries around the world [4-9]. However the taxonomy of *rhizobia* is very diverse [10], molecular techniques based on the Polymerase Chain Reaction (PCR) provided easy and quick methods to microbial characterization [11,12]. The 16S rRNA gene sequencing is an excellent tool for molecular characterization of the different isolates of *Rhizobium* [13,14].

The aims of this study were to characterize *Rhizobium* isolates collected from different Governorates in Egypt and to evaluate their tolerance of some heavy metals.

Materials and Methods

Isolation of Rhizobia

Eleven rhizobium isolates were collected from root nodules on broad bean (*Vicia faba* L.) plants from 11 different fields representing different geographic sites in Egypt according to the methods described by Vincent 1970. Table 1 and Figure 1 show the isolation sites and the name of the isolates.

DNA extraction

Total genomic DNA was extracted from bacterial cultures grown in Yeast Extract Mannitol media (YEM) as described by Shamseldin et al. [9]. The quality and quantity of DNA was characterized both spectrophotometrically and by 0.8% agarose gel. The DNA from all isolates produced clear sharp bands, indicating good quality of DNA. Samples were then diluted to 20 ng DNA μL^{-1} and kept at -20°C.

Amplification of 16S rRNA gene

The DNA of the Rhizobium isolates was amplified using the universal primers, fD1 (5' AGAGTTTGATCCTGG CTCAG 3') and rP2 (5' ACGGCTACCTTGTTA CGACTT 3') as described in Tsuzuki et al., [15]. The PCR reaction was performed in 50-µL reaction volume containing 100 ng DNA, 25 µL Maxima Hot Start PCR Master Mix (Fermentas, Lithuania) and 20 µM of forward and reverse primers. Amplifications were performed with the following conditions: initial denaturation at 95°C for 10 min, 35 cycles of 95°C for 30 s, 58°C for 1 min, 72°C for 1 min and 10 min final extension at 72°C.

Partial 16S rRNA gene sequencing

The PCR products for the eleven isolates were used in sequencing of the 16S rRNA gene from both strands using the same primers used in PCR amplification and Big Dye Terminator DNA analyzer (ABI) at Bioneer (Daejeon, Korea).

Evaluation of heavy metals tolerance

The eleven Rhizobium isolates were evaluated for their tolerance against three different heavy metals (Cu, Zn and Pb) on solid YEMA medium. The stock solutions of heavy metals (mM) were filtered, sterilized and added to sterile agar as follows: $CuCl_2.2H_2O$ 0.5, 1 and

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2; $ZnSO_4.7H_2O$ 0.5, 1 and 2; $Pb(C_2H3O_2)_2.3H_2O$ 0.5, 1.0 and 2.0. The plates were inoculated with 10^{-8} cells and the bacterial growth was evaluated after 7 days at 28°C [16,17]. Isolates were considered resistant Isolates were considered resistant if growth was observed or sensitive if otherwise.

Statistical analysis

Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 6 [18].

Results and Discussion

Amplification and sequencing of 16S rRNA gene

This work presents study on the genetic diversity of 11 *Rhizobium leguminosarum symbiovar. Viciae* isolates collected from different V*icia faba* fields and representing several governorates in Egypt (Table 1 and Figure 1).

The 16S rRNA gene sequencing, which is widely used for molecular phylogeny of rhizobia [13,14,19], was used as powerful tool to study the diversity and phylogenetic of Rhizobium isolates. The 16S rRNA gene was amplified using fD1 and rP2 primers as described by Tsuzuki et al. [15], and all the isolates yielded a single-fragment about 850 bp. After amplification the same primers were used for partial sequencing of 16S rRNA region from the both sides. The alignment was done for all isolates sequences and the genetic diversity was estimated based on the number of base pairs substitution per site among all isolates (Table 2). The phylogenetic relationship analysis according to these data divided the isolates into two principal groups (Figures 2 and 3); the first group included isolates numbers 1,2,3,4,5,7,8,9,10 and 11 which were isolated from Menoufia, Menoufia, Gharbia, Gharbia, Gharbia, Behira, Dakhalia, Giza, Sharkyia and Ismailia governorates respectively. While the second group included only one isolate RL6 which was isolated from North Sinai Governorate. The genetic distance values between the isolates were variable and ranged from 0.02 to 0.8. The lowest genetic distance values were obtained between isolate RL9 of Giza and RL10 of Sharkyia; the isolates RL4 of Gharbia and RL9 of Giza; the isolates RL1 of Menoufia and RL9 of Sharkyia with genetic values of 0.02, 0.03 and 0.05 respectively. While the highest genetic value was between RL6 of North Sinai and RL8 of Dakhalia.

It should be mentioned that the genetic distance values between isolates collected from different fields but from the same governorate for example Menoufia and Gharbia are low. In general, the values of genetic distances among the first group isolates are low. This may be due to the high conserved nature of the 16S rRNA gene sequences, so the sequences variability are limited and, second, the distance between the isolation sites of first group isolates ranged from 30 to 130 km so that it is possible that these isolates has originated from the same genetic background and the human activities like soil and plant transfer limited the genetic diversity of these isolates.

The second group included the isolate RL6 of north Sinai governorate which are located about of 300 km from isolation sites of other isolates and this may give explanation for the high genetic distances values with these isolates.

Evaluation of heavy metals tolerance

All of Rhizobium isolates were tested for their tolerance to heavy metals using concentrations of 0.5, 1 and 2 mM of heavy metals (Cu, Pb and Zn). The isolates can be considered tolerant when the growth occurs in the presence of heavy metals. At the low concentration (0.5 mM), 73%, 100% and 91% of isolates were found to be resistant of Cu, Pb and Zn respectively. Moreover at the concentration of 1mmol, 37%, 80% and 55% of isolates were found to be tolerant of Cu, Pb and Zn respectively. The resistance of *Rhizobium* isolates was recorded for copper, zinc and lead at concentration of 0.5, 1 mM of Cu and Zn. this result is consistent with previous literature which indicated that Rhizobium is resistant to high concentrations of arsenate, zinc, copper, and mercury [20-22]. The ability to resist the heavy metals decreased with increase in concentration. At the highest concentration (2 mM), No growth was obtained with addition of Zn and Mn, however only 27% of isolates could survive with the same concentration of Pb. Previous studies have shown that the increased concentrations of heavy metals can affect the growth, morphology and activities of microorganisms, including symbiotic N_2 fixation [23-26]. Resistance of some tested isolates for the highest concentration of Pb is not understood [27]. One of explanations is that these isolates were isolated from soil polluted with Pb and have probably adapted to this environmental stress [28-31].

In general, the studied isolates showed a variable resistance against heavy metals and this will allow selecting of good candidates for genetic studies.

Rhizobium isolates	Source
RL1	*Menoufia governorate*
RL2	*Menoufia governorate*
RL3	*Gharbia governorate*
RL4	*Gharbia governorate*
RL5	*Gharbia governorate*
RL6	*North Sinai governorate*
RL7	*Behira governorate*
RL8	*Dakhalia governorate*
RL9	*Giza governorate*
RL10	*Sharkya governorate*
RL11	*Ismailia governorate*

Table 1: Rhizobium isolation governorates

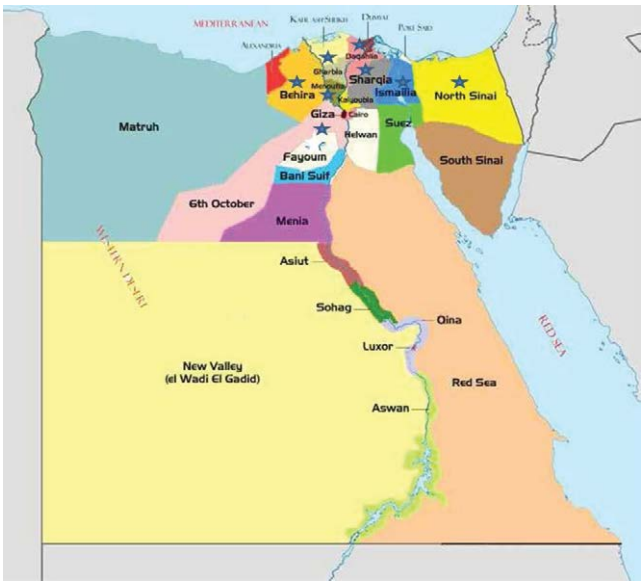

Figure 1: Map show distribution of Rhizobium isolation sites in Egypt

RL11	RL10	RL9	RL8	RL7	RL6	RL5	RL4	RL3	RL2	RL1	Isolates
										0.0	RL1
									0.00	0.26	RL2
								0.00	0.12	0.27	RL3
							0.00	0.24	0.27	0.08	RL4
						0.00	0.25	0.04	0.14	0.28	RL5
					0.00	0.61	0.53	0.59	0.53	0.52	RL6
				0.00	0.6	0.21	0.26	0.2	0.22	0.29	RL7
			0.00	0.34	0.8	0.22	0.27	0.24	0.28	0.25	RL8
		0.00	0.31	0.25	0.54	0.25	0.03	0.24	0.24	0.05	RL9
	0.00	0.02	0.28	0.26	0.53	0.24	0.03	0.24	0.26	0.07	RL10
0.00	0.38	0.36	0.3	0.37	0.71	0.2	0.4	0.21	0.31	0.38	RL11

Table 2: Number of base substitution per site between 11 *Rhizobium legurninosarum* isolates as obtained by Mega 6

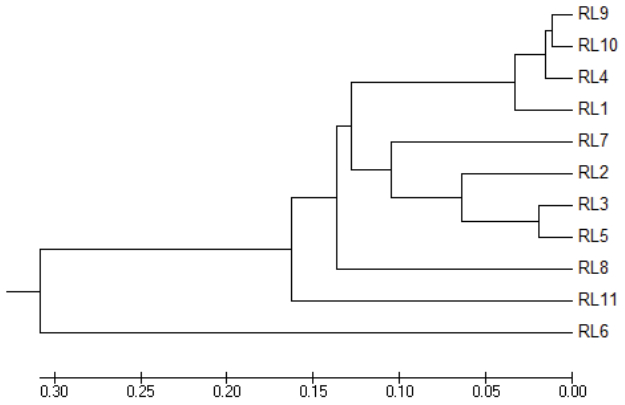

Figure 2: Phylogenetic relationship between 11 Rhizobial isolates using MEGA6 program based on 16S rRNA gene sequence data

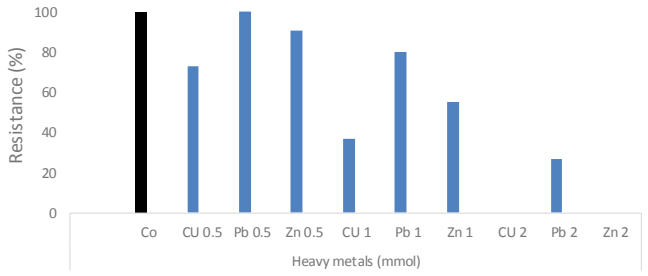

Figure 3: Resistance of Rhizobial isolates against heavy metals concentrations (0.5, 1 and 2 mM) as compared with control (Co)

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