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By

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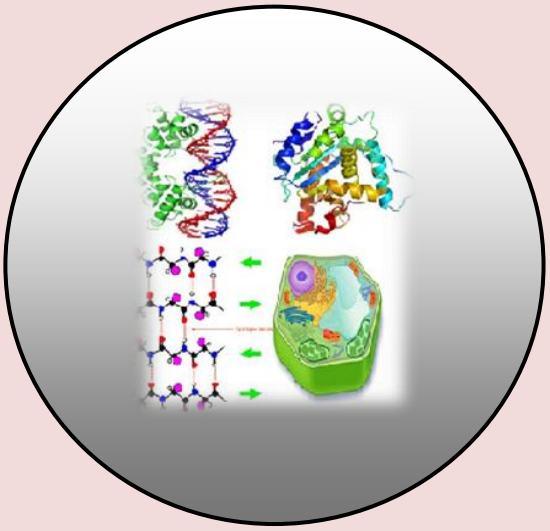
ISSN 2319-3077 Online/Electronic
ISSN 0970-4973 Print

UGC Approved Journal No. 62923
MCI Validated Journal
Index Copernicus International Value
IC Value of Journal 82.43 Poland, Europe (2016)
Journal Impact Factor: 4.275
Global Impact factor of Journal: 0.876
Scientific Journals Impact Factor: 3.285
InfoBase Impact Factor: 3.66

J. Biol. Chem. Research
Volume 36 (1) 2019 Pages No. 45-51



Journal of Biological and Chemical Research

An International Peer Reviewed / Referred Journal of Life Sciences and Chemistry

Indexed, Abstracted and Cited in various International and National Scientific Databases

Published by Society for Advancement of Sciences®

J. Biol. Chem. Research. Vol. 36, No. 1: 45-51, 2019
(An International Peer Reviewed / Refereed Journal of Life Sciences and Chemistry)
Ms 36/01/50/2019

ISSN 2319-3077 (Online/Electronic)
ISSN 0970-4973 (Print)

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RESEARCH PAPER

Received: 09/11/2018 **Revised: 14/01/2019** **Accepted: 15/01/2019**

Bacterial Diversity in Nodules and Rhizosphere of a Chickpea (*Cicer arietinum* L.) Grownin Saline and Non-Saline Soils

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ABSTRACT

Chickpea is one of the most important food legumes, however it is very sensitive to soil salinity. Salinization impairs chickpea growth and influence on all its parameters. At the same time salinity decrease bacterial diversity in its nodules and rhizosphere. The aim of this research was to define the species of bacteria which dwell in chickpea nodulesand rhizosphere in salinity conditions and compare them to bacterial diversity in non-saline soils. The total soil and nodules DNA was isolated and 16S rRNA genes amplification was carried out. DGGE analysis allowed us to separate the PCR products to bands and bands were cut off, DNA purified, reamplified and identified after sequencing. The results showed that bacterial diversity in nodules and rhizosphere of chickpea grown in non-saline soil (EC-313 mSm^{-1}) is rich as compared to saline (EC-659 mSm^{-1}). 27 bacterial strains related to different species were isolated from samples of non-saline soils and only 13 from saline. Comparing the identified species to world literature data it is supposed that more of the strains could be potential PGPR. In spite of less number of bacteria dwelling chickpea nodules and rhizosphere these salt-tolerant bacteria are essential for chickpea survival in saline soil.
Key words: Chickpea, Bacteria, Diversity, DGGE and Salinity.

INTRODUCTION

Chickpea (*Cicer arietinum* L.) is one of the main food legume cultures, and also an important source of protein in many countries, however as well as many other legumes, chickpea is very sensitive to soil salinization (Maasand Hoffman, 1977). The deleterious effects of salinity on plant growth are associated with water stress (low osmotic potential of soil solution); nutrient ion imbalance; salt stress (specific ion effects); and a combination of all these factors (Ashraf and Harris, 2004). All these factors cause adverse pleiotropic effects on plant growth and development at physiological, biochemical, molecular and whole plant levels. Salinity reduces plant height, leaf number, leaf size, stem and root dry weights, and seed emergence (Esechie et al., 2002; Welfare et al., 2002). The same factors affect microorganisms' activity and are the key point for natural selection of the most tolerant bacteria strains able to overcome these conditions (Borneman et al., 1996). There are some reports about single bacteria found in a chickpea nodules and rhizosphere, which can improve its growth in salinity conditions (Hamaoui et al., 2001; Mishra et al., 2010). However there is no data about full composition of bacterial community in a chickpea rhizosphere in salinization conditions in the world literature. The purpose of our research was study and comparison of bacterial community composition in nodules and rhizosphere of a chickpea growing in saline and non-saline soils for formation of a specific vision about influence of salinization on bacterial diversity in chickpea nodules and rhizosphere.

MATERIAL AND METHODS

Samples collection and DNA extraction

Chickpea rhizosphere soil samples were collected in July. Soil and chickpea roots samples were taken from two different chickpea fields of Syrdarya region of Uzbekistan. One of the fields was with saline soil (EC-659 mSm^{-1}) and another with non-saline (EC-313 mSm^{-1}) (Table 1). According to Rhian et al. (2002), soils with EC (electrical conductivity) higher than 400 $mSm^{-1}$are considered as saline. We used 3 sources of samples which are the soil close to rhizosphere, rhizosphere and chickpea nodules. Plants with the soil close to roots were collected from five places of each field, first sample from the centre and 4 from the corners. Then each sample was treated as follows. The soil close to rhizosphere was scraped off from roots but without touching the roots surface, then mixed with same samples taken from one field and used for DNA extraction. To get rhizosphere samples the roots were gently shaken from stuck soil particles by hand and then were cut to 3 cm pieces. The pooled root pieces were added to 1000 ml Erlenmeyer flask, containing 250 ml of sterilized water and shaken for 30 min. The soil suspension was centrifuged at 4000 rpm for 10 min, and the precipitate was collected as rhizosphere soil and used for DNA extraction. To get third sample source – chickpea nodules were cut off from the roots, surface-sterilized using 70 % ethanol and repeatedly washed with sterile water. Sterile nodules were crushed with the sterile blade. Crushed nodules from roots of 5 plants of each field were mixed together and used for DNA extraction. Total soil and nodules genomic DNA was extracted using the Power Soil DNA Isolation Kit following the manufacturer's instructions. The concentration and quality of the DNA were determined using a spectrophotometer Nano Drop.

Table 1. Chemical characteristics of soil samples.

Soil type	Depth (cm)	E (mSm^{-1})	Organic matter (%)	Total C (%)	Total N (%)	Ca^{+} (g/kg)	Mg^{+} (g/kg)	K^{+} (g/kg)	P^{-} (g/kg)	Cl^{-} (g/kg)	Na^{+} (g/kg)	pH
Saline	0-10	659	0,83	3.154	0,103	69.3	25.4	9.4	1.6	0.4	2.9	8.01
	10-20	623	0.69	2.506	0.091	63.5	20.7	7.2	1.2	0.3	2.1	7.83
	20-30	565	0.62	2.122	0.078	51.2	16.3	6.5	1.1	0.3	1.9	7.76
	30-40	492	0.51	1.535	0.062	46.8	14.5	5.7	1.0	0.1	0.7	7.54
Non saline	0-10	313	1.52	5.678	0.196	42.5	16.2	15.3	2.9	0.2	1.3	7.58
	10-20	274	1.44	5.103	0.181	34.9	12.7	12.8	2.7	0.1	0.7	7.47
	20-30	221	1.27	4.337	0.173	28.6	10.6	11.6	2.6	0.1	0.6	7.32
	30-40	143	1.12	3.565	0,159	23.7	9.5	10.4	2.4	0.1	0.6	7.27

16S rRNA analysis

The DNA extracted from each sample served as a template for bacterial 16S rRNA genes. 16S rDNA was amplified by polymerase chain reaction (PCR) using universal forward 16SF: 5'-GAGTTTGATCCTGGCTCAG-3' and reverse 16SR: 5'-GAAAGGAGGTGATCCAGCC-3' primers (Mohanta et al., 2015). PCR mixes with a volume of 25µl contained 5µl 5× buffer, 1 µl of 2% BSA, 0.5µl dNTP mixture (10 mM of each dNTP), 0.5 µl ofeach primer (10 µM), 0.125 µl OneTaq DNA Polymerase, 15.375µlmq water and 2µl of template DNA. The PCR program started from denaturation for 30 s at 94°C. Afterwards, 25 cycles with 17 s 94°C, 35 s 55°C and 90 s 68°C followed. Then elongation during 25 min at 68°C. The PCR products were verified by gel electrophoresis on 1.0 % agarose gel on TAE buffer.

DGGE analysis

Denaturing gradient gel electrophoresis was done by means of D Code system. Equal amounts of PCR products (6 µl) were loaded onto 8% acrylamide gels with a denaturing gradient of 30-60% (where 100% denaturing is defined as 7 M urea and 40% form amide (Muyzer et al. 1993) for optimal separation of the PCR products. DGGE gels were run during 20 h at 65 V and 60 ^{0}C in 0.65 TAE buffer and colored for 30 min with SYBR Gold. Gels were visualized using the transilluminator. Bands were cut off from gel and stored for further processing.

Sequencing of the DGGE bands

DGGE bands were prepared for sequencing according to Sanguinetti et al. (1994). 2 µl of the extracted DNA solution were reamplified with the same primers as for 16S rRNA analysis. PCR amplicons were purified using Exo SAP-IT kit. Sequencing was performed by Sequence Laboratory by standard Sanger method. Sequences were aligned and compared with Gen Bank database using Blast N program (NCBI).

RESULTS AND DISCUSSION

We have studied the samples of soil close to rhizosphere, rhizosphere and nodules of chickpea growing in saline and non-saline soil. It was isolated the DNA and carried out the amplification of 16S rRNA genes from these samples. As a result of DGGE analysis and sequencing of DNA of the samples taken from non-saline field 27 bacteria strains of various species (table 2) are isolated and identified.

It is apparently from table 2, that the majority of isolated strains are presented by species of genuses *Pseudomonas* (7 strains) and *Bacillus* (7 strains), and also 2 species of *Azotobacter*, 2 species of *Azospirillum*, 3 species of *Mesorhizobium* and others. Thus some of isolated bacteria can be potential PGPR. So for example it is known, that many strains of *Pseudomonas* improve nodule formation at chickpea, and also enhance growth and development of chickpea by means of biocontrol (Sindhu and Dadarwal, 2001; Kumar et al., 2001), phosphate mobilizing properties (Rosas et al., 2006), and also phytohormones production (Joseph et al., 2007). *Azotobacter* strains could affect seed germination and seedling growth (Shaukat et al., 2006) in a plant by means of nitrogen fixation, IAA production (Joseph et al., 2007), siderophores production and phosphates solubilization (Husen, 2003). Members of the genus *Azospirillum* fix nitrogen under microaerophilic conditions and are frequently associated with root and rhizosphere of a largenumber of agriculturally important crops and cereals (Bashan et al., 2004). *Bacillus* is the most abundant genus in the rhizosphere, and the PGPR activity of some of these strains has been known for manyyears, resulting in a broad knowledge of the mechanisms involved (Probanza et al., 2002; Gutiérrez Mañero et al., 2003) like phosphate mobilization, siderophore and antifungal metabolite production resulting in biocontrol of phytorathogenic fungi (Chakraborty et al., 2006). Non-symbiotic nitrogen fixation has a great agronomic significance. *Achromobacter* and *Artrobacter* are related to non-symbiotic nitrogen-fixing bacteria (Saxena and Tilak, 1998). It was reported that *Arthrobacter ureafaciens* was an active tricalcium phosphate solubilizer resulting in plants growth stimulation (Chen et al., 2006). *Mesorhizobium ciceri* and *Mesorhizobium mediterraneum* are two special nodulating symbionts of a chickpea, are known as good phosphate solubilizers (Rivas et al., 2006). It was reported that rhizosphere competent *Mesorhizobiumloti* MP6 produces hydrocyanic acid (HCN) under normal growth conditions and enhances the growth of Indian mustard (*Brassica campestris)* (Chandra et al., 2007). Rhizobial isolates belonging to genera *Rhizobium* sp. and *Mesorhizobium* sp. produces catecholate type of siderophores (Joshi et al., 2009). These properties are important for biocontrol of phytopathogenic fungi.

Such a wide diversity of various bacteria species can be explained by rich inhabitancy, i.e. a soil compound. Apparently from table 1, the soil of non-saline field is rich with humus, carbon, nitrogen, phosphorus and potassium. These elements are essential both for plants growth, and for microorganisms' growth. Also the soil rich with microelements and absence of a salinization allows chickpea to develop actively, thus chickpea roots secrete considerable amount of exudates which also provide life activity of bacterial community in its nodules, rhizosphere and soil close to rhizosphere. Unlike these results from saline field samples only 13 strains of bacteria (Table 3) are secreted. All of them are identified by means of algorithm BLAST. Results show that in investigated samples bacteria of genus Pseudomonas (5 strains) and Bacillus (4 strains) are dominating.

The bacterial community in samples from saline soils was presented with salt-tolerant strains, some of which are capable to improve chickpea growth in salinization conditions. Thus according to Egamberdieva (Egamberdieva et al., 2013a) salt-tolerant strain *Pseudomonas extremorientalis* TSAU20 was able to stimulate growth of *Silybum marianum* under salt stress. It was reported that strain *Pseudomonas pseudoalcaligenes* isolated from rice field was able to solubilize phosphates, produce siderophore, indoleacetic acid (IAA) and gibberellin and utilize ACC (1-aminocyclopropane-1-carboxylate) as sole nitrogen source (Jha and Subramanian, 2014). It was found out that *Pseudomonas aeruginosa* P66 actively produced HCN, *P. aeruginosa* P112 showed high IAA production, *P. aeruginosa* P12 and Bacillus subtilis B28 isolates were the most effective in reduction of chickpea fusarium wilt (Karimi et al, 2012). The results of Han et al. (2014) are indicate that soil inoculation with Bacillus subtilis GB03 promotes white clover growth under both non-saline and saline conditions by directly or indirectly regulating plant chlorophyll content, leaf osmotic potential, cell membrane integrity and ion accumulation. Akhtar et al. (2010) reported that lentil inoculation with *B. pumilus* together with *P. alcaligenes* caused a great increase in plant growth, number of pods, nodulation, and root colonization byrhizobacteria, and also reduced *Fusarium* wilt on lentil. Results of Qureshi et al. (2009) revealed that *Mesorhizobium ciceri* and *Bacillus megaterium* at coinoculation significantly increased the yield of chickpea as compared to control. Halotolerant *Azospirillum halopraeferens* was discovered in 1987 (Reinhold et al. 1987). It is known that these bacteria are nitrogen fixing and according to reports (Puente, 1999; Reinhold et al. 1987) associated with the roots of *Leptochloafusca* and black mangrove.

Table 2. The results of sequence analysis of the dominant 16S rRNA gene amplicons isolated from total DNA of non-saline soil samples.

DGGE band No.	Source of strain isolation	Most closely related microorganism	Similarity (%)	Accession No. of related sequence
1	Soil close to rhizosphere	*Pseudomonas fluorescens* strain FW300-N2C3	99	NZ_CP012831.1
2	Rhizosphere	*Pseudomonas rhizosphaerae* strain DSM 16299	98	NZ_CP009533.1
3	Rhizosphere	*Pseudomonas putida* KT2440	99	NC_002947.3
4	Rhizosphere	*Pseudomonas chlororaphis* strain PA23	99	NZ_CP008696.1
5	Soil close to rhizosphere	*Pseudomonas syringae* pv. *Syringae* B301D	98	NZ_CP005969.1
6	Soil close to rhizosphere	*Pseudomonas aeruginosa* strain F9676	99	NZ_CP012066.1
7	Rhizosphere	*Pseudomonas stutzeri* CCUG 29243	98	NC_018028.1
8	Soil close to rhizosphere	*Azotobacter chroococcum* NCIMB 8003	100	NZ_CP010415.1
9	Soil close to rhizosphere	*Azotobacter beijerinckii* strain ICMP 8673	99	NR_042071.1
10	Soil close to rhizosphere	*Azospirillum brasilense* Sp 245	98	NC_016617.1
11	Soil close to rhizosphere	*Azospirillum lipoferum* 4B	99	NC_016622.1
12	Rhizosphere	*Bacillus cereus* ATCC 14579	100	NC_004722.1
13	Rhizosphere	*Bacillus circulans* strain RIT379 contig72	98	NZ_LDPH01000072.1
14	Soil close to rhizosphere	*Bacillus lichen iformis* ATCC 14580	99	NC_006270.3
15	Soil close to rhizosphere	*Bacillus megaterium*DSM319	99	NC_014103.1
16	Soil close to rhizosphere	*Bacillus mycoides* strain B38V scaffold76.1	99	NZ_JYFS01000099.1
17	Rhizosphere	*Bacillus subtilis* strain UD1022	100	NZ_CP011534.1
18	Soil close to rhizosphere	*Bacillus pumilus* strain 7P scaffold00001	99	NZ_JHUD02000001.1
19	Rhizosphere	*Paenibacillus polymyxa* strain KF-1 scaffold00032	97	NZ_LNZF01000032.1
20	Soil close to rhizosphere	*Clostridium pasteurianum* NRRL B-598	98	NZ_CP011966.1
21	Rhizosphere	*Arthrobacter globiformis* NBRC 12137	99	NZ_BAEG00000000.1
22	Rhizosphere	*Agrobacterium tumefaciens* WRT31	99	NZ_CM002024.1
23	Nodules	*Mesorhizobium ciceri* biovar *biserrulae* WSM1271	100	NC_014923.1
24	Nodules	*Mesorhizobium mediterraneum* strain LMG 17148	99	NR_042483.1
25	Rhizosphere	*Mesorhizobium loti* MAFF303099	99	NC_002678.2
26	Rhizosphere	*Achromobacter xylosoxidans* A 8	99	NC_014640.1
27	Nodules	*Ochrobactrum ciceri* strain Ca-34	99	NR_115819.1

All these reports show the plant growth promoting and biocontrol properties of different bacteria that are the same species as ours. The results show that bacterial diversity in nodules and rhizosphere of chickpea grown in saline soils issharply restricted because of salinization conditions and low content of nutritive elements (N, P, K) and organic matter that confirm the report of Borneman (Borneman et al., 1996).

Table 3. The results of sequence analysis of the dominant 16S rRNA gene amplicons isolated from total DNAof saline soil samples.

DGGE band No.	Source of strain isolation	Most closely related microorganism	Similarity (%)	Accession No. of related sequence
1	Rhizosphere	*Pseudomonas extremorientalis* strain KMM 3447	99	NR_025174
2	Rhizosphere	*Pseudomonas pseudoalcaligenes* CECT5344	99	NZ_HG916826.1
3	Rhizosphere	*Pseudomonas alcaligenes* NBRC 14159	98	NZ_BATI01000076.1
4	Soil close to rhizosphere	*Pseudomonas aeruginosa* strain F9676	97	NZ_CP012066.1
5	Rhizosphere	*Pseudomonas chlororaphis* strain PA23	99	NZ_CP008696.1
6	Rhizosphere	*Bacillus cereus* ATCC 14579	99	NC_004722.1
7	Rhizosphere	*Bacillus subtilis* strain UD1022	98	NZ_CP011534.1
8	Soil close to rhizosphere	*Bacillus megaterium* DSM319	98	NC_014103.1
9	Soil close to rhizosphere	*Bacillus pumilus* strain 7P scaffold00001	99	NZ_JHUD02000001.1
10	Nodules	*Mesorhizobium ciceri* biovar *biserrulae* WSM1271	100	NC_014923.1
11	Soil close to rhizosphere	*Serratia marcescens* subsp. *marcescens* Db11	97	NZ_HG326223.1
12	Soil close to rhizosphere	*Azospirillum halopraeferens* DSM 3675 G472DRAFT_scaffold00008.8_C	99	NZ_AUCF01000010.1
13	Soil close to rhizosphere	*Clostridium pasteurianum* NRRL B-598	98	NZ_CP011966.1

CONCLUSION

From the present study we can conclude that in non-saline soils with the high content of nutrients in a chickpea rhizosphere dwells a big number of various bacteria species, many of which can perform similar functions such as nitrogen fixation, phosphate solubilization, production of phytohormones, siderophores etc. However in saline soil persist only not numerous salt-tolerant bacterial strains and they allow chickpea to overcome salt stress and grow in salinization conditions (Upadhyay et al., 2011, Egamberdieva et al., 2013b). In turn, the chickpea secreting root exudates, sustains bacterial activity. Thus, interactions of bacterial community with chickpea are more interdependent in the conditions of a salinization, than in not saline soils.

ACKNOWLEDGEMENTS

This work has been financed by the Ministry of Innovational Development of the Republic of Uzbekistan.

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Title should be "Sugarcane in Asia-Climate Change and Drought".

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