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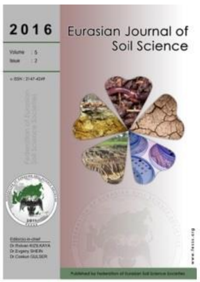

Effect of chickpea in association with *Rhizobium* to crop productivity and soil fertility

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Abstract

The growth, development and yield of chickpea (*Cicer arietinum* L.) is strongly influenced by abiotic factors such as salinity and drought in the arid conditions. The use of efficient plant growth promoting bacteria in chickpea production is the best solution to overcome those stresses. In the present study, 10 chickpea rhizobial strains were isolated and purified from the nodules of chickpea genotype grown on middle salinated soils with different chickpea cultivation histories, 3 of them were more efficient in salt tolerance and showed higher nodulation abilities. Local chickpea genotype Uzbekistan-32 was inoculated with selected *Rhizobium* bacterial strains before planting them to the field condition. Inoculation of plants with strains *Rhizobium* sp. R4, R6 and R9 significantly increased shoot, root dry matter, and nodule number by 17, 12, and 20% above the uninoculated plants, respectively. The shoot length increased by 52%, root length by 43%, shoot dry weight by 36%, and root dry weight by 64%. Inoculation significantly increased the pod number by 28% and yield up to 55% as compared to control plant. The effective indigenous rhizobial strains isolated in this study from chickpeas on middle salinated soils of Uzbekistan have the characters of broad host range, high nodulation efficiency, efficient N fixation, great salt tolerance. Soil nitrogen, phosphorus and carbon content of the soil at the end of experiments were positive in all the treatments compare control. In this study, we are focused with consideration of the relationship between chickpea and its symbiotic nitrogen-fixing root nodule bacterial strains and how it functions to influence plant productivity and soil fertility.

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Introduction

Chickpea (*Cicer arietinum* L.) is a very important legume food crop in arid regions considering as an essential source of protein, lipid, carbohydrates and vitamins for human beings. However, chickpea production is severely limited in arid areas of Uzbekistan due to soil salinity and drought condition. Also, low soil fertility and harsh climate are the important factors limiting legume production.

Uzbekistan is one of the countries most seriously affected by land degradation and desertification in the world, as is evidenced by the 85% of the land that now suffers from various levels of secondary salinization (Gintzburger et al. 2003). It limits the area under of cereals and food legumes cultivation, hence affects food security. It is further reported that approximately 20000 ha of irrigated land in Uzbekistan are lost to salinity

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and invariably abandoned every year (Toderich et al. 2009). There is urgent need to take preventive steps to overcome the current conventional agricultural approach to make agriculture in irrigated drylands sustainable. The greatest challenge to our arable agriculture in the long term is the maintenance, and preferably the improvement of soil quality.

Legume cropping systems that increase soil fertility and concurrently enhance plant productivity and prevent erosion and desertification are of major interest in many countries in the world (Egamberdieva et al. 2014). Incorporating of legume crops in crop rotation system increases the yield of both cotton and wheat which are the main crops grown in Uzbekistan occupying 80% of the total irrigated area, but crops yield have been decreasing due to worsening soil conditions (Khaitov et al. 2014).

Chickpea is usually grown on the marginal land. With the function of biological N fixation in association with rhizobial strains, chickpea could be considered as an excellent rotation and intercropping crop by improving soil fertility and structure, and decreasing soil erosion in agricultural production system (Shurigin et al. 2015).

Although, legume- *Rhizobium* symbiotic nitrogen (N) fixation is an important biological character and also the base of improving soil fertility, case in point is a lack of effective rhizobial inoculants adapting to salinated soils. Previous studies have shown that salinity and drought stress led to a significant decline in plant biomass accumulation (root and shoot), nodule development, and nitrogenase activity as well as strongly declined the yield in chickpea (Garg and Baher, 2013; Egamberdieva et al. 2014). Chickpea can restore soil fertility due to deep penetrating root system which enables them to utilize the limited available moisture (Tripathi et al. 2015).

There are evidences that certain strains of bacteria in the root nodules of leguminous crops can help tolerate toxic levels of salinity. The salt tolerance abilities of rhizobia may have an important effect on the successful *Rhizobium*-legume associations under salinity condition. It aims at obtaining ecologically safe food and higher yield without disturbance of the environment and simultaneously, improves the soil quality.

In Uzbekistan, the indigenous *Rhizobium* abundance in most soils is very low because of lack chickpea cultivation, and the rhizobial inoculants from chickpea cultivations of different regions could not adapt to salinated soils. Therefore, it is very pivotal to select efficient rhizobial strains, which are well adapted to salinated soils for developing chickpea production and improving chickpea yield and soil fertility.

In this study, a series of rhizobial strains were isolated and purified from salinated soils and local chickpea variety, and their nodulation efficiency were tested both in soil pot and field condition. Furthermore, the inoculated bacterial strains were able to persist in the rhizosphere showing colonization on root and within nodules. Present study shows that plant growth promoting rhizobacteria (PGPR) inoculation should be integrated with chickpea production program in Uzbekistan especially for marginal soils.

Material and Methods

Study site and soil sampling

Soil for pot experiment was sampled from an irrigated agricultural site located in Syrdarya Province (41°00′N, 64°00′E,) in north-eastern Uzbekistan. In these soils, cotton has been grown for the long years under a continuous monoculture production system and under flood irrigation without proper drainage facilities but using a natural flow system. According to the WRB-FAO (2006) classification, the soils of selected fields were identified as Calcisol (silt loam serozem) having a calcic horizon within 50 cm of the surface. Soil basic physical and chemical properties from this site were summarized in Table 3 and 4. The surface soil horizon was calcareous saline whereas the deeper soil horizons were only mildly alkaline. The orchic horizon is low in organic matter. The climate is semiarid with mean annual air temperatures of 16^{o}C and 18^{o}C, and mean annual rainfalls of 180-200 mm. The conventional tillage consisted of moldboard plowing to 30 cm depth after harvest and offset disking, to a depth of 10 cm, prior to planting in the spring. Soil samples of 0-30 cm depth were taken with a soil corer (3.5 cm diameter). Samples were collected at the beginning March (spring), and end of the experiments July (summer). The cores were pooled; field-moist soils were sieved (<2mm) directly after collection. The soil samples were kept in black polyethylene bags and stored at 4°C. These "fresh" field-moist, sieved samples were used for the incubation study. Conventional mineral fertilizers N, P, K input rates range from 200; 140; 70 kg ha^{-1}yr^{-1} respectively in all plots of the experiment.

Plant and microorganisms

Seeds of the chickpea "Uzbekistan-32" were obtained from Seed Production laboratory of Tashkent State Agrarian University. All bacterial strains were previously isolated from the rhizosphere of chickpea grown in salinated soil of Uzbekistan.

Isolation, purification, sequencing and inoculation of Rhizobial strains

Nodules from local chickpea genotype Uzbekiston-32 grown in pots filled with salinated soil brought from field sites in Sirdarya region in Uzbekistan, were used to isolate and purify rhizobial strains. There were a few years of chickpea cultivation history in Sirdarya region's experimental field, where the soil had a higher abundance of indigenous *Rhizobium*, slightly more salinitic. The procedures of *Rhizobium* isolation and purification followed as reported in following method. First, the fresh nodules were washed in 95% ethanol for 3 min, then sterilized in 0.2% Hg_2Cl for 5 min, and rinsed with sterilized water for 5-6 times. After that, we cut the nodules in half on the sterilized slide and used a half nodule to draw lines on the surface of the acid YMA medium. Then the YMA medium was incubated upside down for 6-7 day under 28^0C. The bacterium looked like *Rhizobium* was picked to purify and obtain pure single colonies. After the gram staining, the isolated rhizobial strains were characterized based on the shape of the bacterium under the microscope.

The inoculation experiment was carried out using the specially designed big tube paper culture system. First, the filter paper with a length about 2/3 of the tube was placed into the tube. Each tube was added a 90 mL low N nutrient solution. The sealed tubes were sterilized 30 min under 121^0C.

Salt tolerance of bacterial isolates

In order to determine the optimum salt concentration for growth, bacterial strains were cultured in YEM medium supplemented with different amounts of NaCl: 2%, 3%, 4%, and 5% NaCl (w/v). The growth rate of bacteria isolates was determined with spectrophotometer after 24, 48, 72 hours.

Germination of seeds

The seeds of chickpea were first sorted to eliminate broken, small seeds and then they were surface-sterilized with a solution of 75 mL chloride + 25 mL water for 2–3 min, rinsed thoroughly with distilled water. Surface-sterilized seeds were transferred on paper tissue towels soaked in 0.5 mM $CaSO_4$ and germinated for seven days in a dark room at 25^oC.

Plant growth promotion in pots

For the seed inoculation, *Rhizobium* strains were grown overnight in TY broth. One ml of bacterial culture was pelleted by centrifugation and cell pellets were washed with 1 ml phosphate buffered saline (PBS; 20 mM sodium phosphate, 150 mM NaCl, pH 7.4) and re-suspended into PBS. The suspension used for the inoculation was adjusted to the final concentration of approximately 10^7 CFU mL^1. Uniform seedlings were first placed with sterile forceps into bacterial suspension for 15 minutes and were then transplanted into pots filled with salinated soil (500 g each pot). Two chickpea seedlings were transplanted into each pot, but later one seedling was removed. The pot experiment had two treatments: seeds without bacterial inoculation and seeds inoculated with bacterial strains. Plants were grown at 20 - 26°C during the day and 17 - 18°C at night and after six weeks the shoot and root length and dry matter of chickpea were measured.

Field experiment

The experiment was carried out on a typical salinated soil, without chickpea cultivations in its history, at the field site of experimental station Tashkent State Agrarian University, Tashkent region in Uzbekistan. The experiment was in randomized complete-block design. There were 4 treatments including 3 *Rhizobium* treatments (with inoculation R4, R6 and R9 and without inoculation- control) and local chickpea genotype Uzbekistan-32, 3 replications for each, 12 plots in total. Each plot was 10 m^2. This experiment employed the inoculants isolated from the previous tube study. These three rhizobial strains had the highest nitrogenase activities and used peat and plant ash mixture (1:1, w/w).

Local chickpea genotype Uzbekistan-32 was used in this experiment. The planting density was 60 cmx15 cm. Irrigation, pest and weed control were carried out on the conventional management for chickpea. There were two harvests. The first harvest was at flowering stage. One representative plant for each plot was harvested. The parameters of shoot biomass, N content, plant height, the number of pods, the number of nodules and nodule dry weight were analyzed. The second harvest was at the maturity stage. The plants were harvested according to their maturity time, and then were used to estimate the yield based on the plot yield.

Soil chemical and physical analysis

Air-dried samples were analyzed for the total C, N, P, K and soil humus contents. Soil particle distribution was determined using natrium phosphate. The total carbon content, Ctot, was identified by elementary analysis while total nitrogen, Ntot, content was determined by the Kjeldahl method. The molybdenum blue method determined the total phosphorus content, Ptot, in soil. Potassium, K, was determined using the Flame Photometric Method (Riehm, 1985). The Atomic Absorption Spectrophotometer (AAS) was employed to measure calcium chlorite ($CaCl_2$). Soil pH-value was measured by means of electrometer.

Statistical procedures

Data were tested for statistical significance using the analysis of variance package included in Microsoft Excel 97. Comparisons were done using Student's *t*-test. Mean comparisons were conducted using a least significant difference (LSD) test (P=0.05).

Results and Discussion

Chickpea production in Uzbekistan is very low because there are so many factors in soil limiting chickpea growth, such as salinity, drought and appropriate rhizobial strains. Legumes could fix N biologically, but only after they form nodules with *Rhizobium*. The *Rhizobium*-legume symbiosis has received most attention as they are widely deployed in agricultural practices for sustainability of crop yield and recovery of soil fertility (Egamberdieva et al. 2015).

The use of specific microbes which stimulate plant growth allows a considerable decrease in the use of agrochemicals which are now being used for plant growth stimulation. This will positively affect emergence of seedlings and further growth in soils with a poor structure such as those in Uzbekistan. Micro-organisms in the rhizosphere react to the many metabolites released by plant roots. Their interactions with roots help in nutrient uptake of plants, also the adaptation of plants to adverse soil chemical conditions and susceptibility to disease (Bouhmouch, et al. 2005). Soil beneficial microorganisms have been studied intensively because of their potential impact on agricultural productivity (Davronova, 2013).

The effective indigenous rhizobial strains isolated in this study from chickpeas on middle salinated soils of Uzbekistan have the characters of broad host range, high nodulation efficiency, efficient N fixation, great salt tolerance. Selected strains were able to increase chickpea yield and to reduce the percentage of diseased plants caused by *Fusarium* pathogens in salinated soil.

Forty bacterial strains of *Rhizobium* sp. previously isolated from chickpea root were screened for their salt tolerance abilities. Most of strains where able to growth under 3% NaCl, and only 10 strains were able to tolerate up to 5% NaCl. The results showed that the seed dormancy enforced by salinity (5% NaCl) was substantially alleviated and the germination was promoted by selected rhizobial strains from 54 up to 90% (data not shown). Those 10 strains where taken for further studies on their effect on growth and symbiotic performance of chickpea under salt stress condition. The results of study showed that salt tolerant rhizobial strains stimulated root, shoot growth and nodulation of chickpea affected by salt stress (Table 1).

Table 1. The effect of *Rhizobium* sp. strains on the root, shoot growth and nodule number of chickpea grown under saline soil condition

Bacterial strains	Nodules number	Shoot length (cm)	Root length (cm)	Shoot dry mass (g)	Root dry mass (g)
Control	0	16.7	12.4	0.213	0.081
R 1	12	18.9	14.5	0.224	0.095
R 2	11	19.2	14.2	0.236	0.093
R 3	11	18.6	13.9	0.223	0.092
R 4	20	26.1*	19.0*	0.286*	0.142*
R 5	12	19.5	14.9	0.233	0.094
R 6	15	22.3*	15.7*	0.293*	0.116*
R 7	13	19.3	14.9	0.236	0.095
R 8	10	18.2	13.9	0.217	0.089
R 9	16	21.8*	15.4*	0.294*	0.115*
R 10	14	19.9	15.3	0.234	0.097

* Significantly different from untreated control plants at P<0.05

Selected salt tolerant rhizobia had good symbiotic association with chickpea variety Uzbekistan, which previously selected as salt tolerant cultivar (Egamberdieva et al. 2014).

Without inoculation, no nodules were formed in the tested local chickpea genotype Uzbekistan-32; with inoculation, the nodulation rates in all were 100%. The rhizobial strains R4, R6 and R9 alleviated quite successfully the reductive effect of salt stress on percentage of germination (up to 70%) and seedling growth. Inoculation with rhizobial inoculants not only made many nodules formed, but also increased chickpea shoot and root biomass. The shoot length increased by 38%, root length by 31%, shoot dry weight by 27%, and root dry weight by 42% after inoculation with bacterial strain R4. Similar results were revealed almost all bacterial strains. More considerable result were found when inoculation of chickpea with *Rhizobium* strains R6 and R9, which significantly increased shoot and root dry matter by 37-38% and 43-42% above the uninoculated plants, respectively. Inoculation significantly increased the pod number and pod weight compared to control plant (Table 2). According Kyei-Boahen et al. (2002) and Berger et al. (2006) growth potential of chickpea depends on the rhizobia association and plant genotype which together influencing the symbiotic performance.

Bano et al. (2010) reported that bacterial strains adapted to drought stress was effective in the root-nodule symbiosis and also alleviated decreased growth and yield of chickpea imposed by drought stress. The three best, effective strains R4, R6, and R9 showed high stimulatory effect on the root and shoot growth of chickpea seedlings which further were used for field experiments.

The colonization of root associated beneficial microbes in the rhizosphere is important for their beneficial effect on plant growth, especially under stress soil conditions (Zahran, 2011). It has been also observed that the survival of rhizobia in the plant root and soil is affected by nutrient deficiency, salinity, drought and acidity (Slattery et al. 2004). In earlier report Lowendorf (1980) reported that salinity inhibited survival and proliferation of *Rhizobium* spp. in the soil and rhizosphere, and infection process. This study on survival of salt tolerant R4, R6 and R9 strains in the rhizosphere of chickpea grown under saline soil condition indicates that screening for salt tolerant rhizobial strains are essential to improve symbiotic performance of chickpea under salt stress condition. They are able to stimulate plant growth, alleviate salt stress and survive in the rhizosphere of plant under extreme soil conditions.

Table 2. The effect selected salt tolerant effective *rhizobium* sp. on the growth and yield of chickpea under field condition

Treatments	Nodule number	Nodule weight, g/plant	Root dry weight, g/plant	Shoot dry weight, g/plant	Yield of chickpea, dt/ha
Uzbekistan-32 control	18±1.4	0.15±0.01	1.35±0.4	17.63±1.8	10.5±1.4
Uzbekistan-32+R4	54±5.3*	0.89±0.14	2.18±0.64	20.43±2.4	14.5±1.6
Uzbekistan-32+R6	42±3.5	0.49±0.04	2.22±0.11	21.43±3.1	13.8±0.9
Uzbekistan-32+R9	69±7.4*	0.43±0.03	1.77±0.09	21.33±3.3	17.8±2.7

* Significantly different from untreated control plants at $P<0.05$

A field experiment was carried out by applying rhizobial inoculants R4, R6 and R9, which showed the highest nitrogenase activity on a typical salinated soil of Uzbekistan. Our results showed legumes inoculated with *Rhizobium* could increase the nodule numbers, improve the activity of *Rhizobium* and other soil microorganisms, enhance N fixation and promote root growth, so as to increase the yield of legume plants. But the inoculation effect on yield increase mainly depends on the competition for nodulation of *Rhizobium* and the nitrogen fixation efficiency. In order to improve the *Rhizobium* application effects, the key measure is to select rhizobial strains with well adaptation to local soils, strong competition for nodulation as well as efficient N fixation.

Although inoculation with *Rhizobium* could significantly promote chickpea growth and increase pod number (Table 2), but not plant height (data not shown). Meanwhile, inoculation with *Rhizobium* could significantly affect reproductive growth of chickpeas.

This might be reasoned that inoculation with *Rhizobium* could improve N nutrition, promote vegetative growth, particularly root growth, as well as benefit root uptake from soil in chickpea. Similar observations reported in other studies where inoculation of chickpea with rhizobia increased plant growth, ground dry matter, number of pods, seed yield, and nitrogen fixation under various climatic conditions (Fatima et al. 2008).

Well-structured and deep penetrating root system of chickpea and legume-*Rhizobium* association caused to improve the soil physical and chemical properties (Table 3 and 4). Soil bulk density decreased in the 0-30 cm layer from 0.02-0.04 g cm^{-3}, increased the number of water stable aggregates from 2.4 to 3.5%, soil permeability from 2.1 to 14.3% and soil organic matter (humus) increased for 0.02-0.03%. Total nitrogen, carbon and phosphorous content in the soil increased for 0.022-0.033, 0.054-0.084 and 0.007-0.015 $g.kg^{-1}$, respectively. The results revealed that inoculation of chickpea seeds with *Rhizobium* strains can considerable improve soil chemical and physical properties compare the control variation trials. These results suggest inoculation of chickpea seed with *Rhizobium* bacteria has the beneficial effects to both crop production and soil fertility.

Table 3. Soil chemical analysis

Treatments	Ct, $g.kg^{-1}$	Nt, $g.kg^{-1}$	Pt, $g.kg^{-1}$
At the beginning of experiment	0.823±0.01	0.056±0.012	0.159±0.01
Uzbekistan-32 control	0.877±0.02	0.078±0.006	0.166±0.02
Uzbekistan-32+R4	0.989±0.03	0.087±0.014	0.174±0.015
Uzbekistan-32+R6	0.984±0.01	0.093±0,009	0.179±0.04
Uzbekistan-32+R9	0.907±0.03	0.089±0.011	0.174±0.012

Table 4. Soil physical analysis

Treatments	Soil Bulk Density ($g.cm^{-3}$)		Humus content, %	
	0-30 cm	30-50 cm	0-30 cm	30-50 cm
At the beginning of experiment	1.544±0.04	1.680±0.05	0.975±0.03	0.842±0.04
Uzbekistan-32 control	1.439±0.02	1.581±0.06	0.995±0.06	0.869±0.05
Uzbekistan-32+R4	1.441±0.03	1.580±0.08	0.995±0.07	0.867±0.07
Uzbekistan-32+R6	1.437±0.05	1.581±0.04	0.999±0.11	0.870±0.06
Uzbekistan-32+R9	1.445±0.07	1.584±0.1	1.005±0.07	0.872±0.08

The total C, N and P concentrations in soil under conventional tillage system depended on chickpea association with rhizobial strains (Table 3). The growing of chickpea genotypes without inoculation with *Rhizobium* had significant effect on soil fertility compare with *Rhizobium* inoculated ones. Inoculation of chickpea genotypes with *Rhizobium* strains have led to increase soil carbon, nitrogen and phosphorous contents. Lower concentration of organic matter, N and P content under chickpea grown without *Rhizobium* can depend on the reduced microbial activity consequently the reduced C input to soil. According Schimmel and Bennett (2004) N mineralization by soil microbes is the key event in the N cycle making mineral N bio-available, whereas plants only uptake mineral N. Changes in N dynamics in soils are closely connected with altering in microbial activities involved in N cycle by biotic and abiotic factors (Aziz et al. 2011). It has been reported that accumulation of N by chickpea and with association *Rhizobium* strains have a substantial effect on the soil organic matter, but also on microbial activities under stressed condition (Kantar et al. 2007). We have observed that soil nutrients were increased under chickpea grown in association with *Rhizobium* strains R4, R6 and R9. It is most probably related to greater release of exudates and availability of N and C substrates, due to legumes extensive rooting system (Egamberdieva et al. 2015), and the availability of mineral nutrients in soil which are of considerable importance to increasing microbial populations (Khaitov and Allanov, 2014). Chickpea had a versatile capacity to produce greater root exudates and enrich the soil with nitrogen through nitrogen-fixing activities (Tripathi et al. 2015).

Results from the field experiment indicated that rhizobial strains isolated by us could infect more chickpea genotypes with effective N fixation and adaptability to salt and drought conditions. It is very meaningful to widely apply the effective rhizobial inoculants in Uzbekistan, in order not only to increase host N nutrition, improve crop production, increase soil N content and fertility, but also effectively reduce applications of chemical fertilizers, particularly N fertilizers, finally aiming for improving the quality of ecological environment, benefit the maintenance of ecological balance.

Conclusion

Most irrigated fields in Uzbekistan have salinated soils. Accelerated secondary salinization of irrigated soil is recognized as the major agricultural problem in Uzbekistan and the substantial areas under crop production in the country are affected by different level of soil salinity.

The abundance of indigenous *Rhizobium* in these areas is very low because of the lack of chickpea cultivation, the high temperature in summer and cold weather in winter seasons. Inoculation with *Rhizobium* is an effective approach to strengthen N fixation, increase N nutrition and promote yield in chickpea. Therefore, inoculation with the effective rhizobial inoculants might be an important approach to improve chickpea production on salinated soils in Uzbekistan. This study revealed that screening for salt tolerant rhizobial strains are essential to improve symbiotic performance of chickpea under salt stress condition as well as improve soil fertility. Application of inoculation techniques with rhizobial inoculants in legume production has great economical, environmental and ecological benefits. Furthermore, the study shows the potential of phytohormone producing strains R4, R6 and R9 as promising candidate for development of biofertilizer along with nodulating strains to get sustainable yield of chickpea with minimum inputs at marginal land.

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