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Phenotypic characterization of indigenous rhizobia nodulating chickpea in Turkey reveals high diversity

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ABSTRACT

A total of 120 root-nodule bacteria from chickpea plants (*Cicer arietinum* L.) from six regions in Turkey were characterized using 71 phenotypes. Utilization of carbon and nitrogen sources, tolerance to salt stress, temperature and pH, resistance to antibiotics and heavy metals and ability to produce some enzymes, were assessed. 90% of the isolates produced mucously, circular, smooth-margined and watery to creamy colonies with 2–4 mm diameter after 1-3 days of growth. The isolates utilized different compounds as sole carbon and nitrogen sources, endured 2% salt, grew optimally at 25-35°C and pHs between 6-8, exhibited insensitivity to the heavy metals Zn, Hg, and Cu and the antibiotics kanamycin, streptomycin and tetracycline. Numerical analysis separated the isolates into three clusters at 25% similarity. Results revealed diversity among isolates and were consistent with previous findings on chickpea Mesorhizobia.

Key words: Chickpea, Diversity, Phenotypic characterization, Rhizobia.

INTRODUCTION

Nitrogen is essential for plant growth and development. It is provided to cropping systems as industrial fertilizers to promote production. Fertilizers cause worldwide ecological and human health problems and hence there is a growing interest in sustainable and secure food production. Biological nitrogen fixation (BNF) is a process whereby atmospheric nitrogen is reduced to ammonia in root nodules of legumes. It contributes approximately 16% of total nitrogen input in cropland and presents the potential to reduce the manufactured N fertilizers. Rhizobia form nodules on legumes where BNF occurs and provides most of the fixed nitrogen to plants. Legumes can grow in arid and N- deficient soils, acting as pioneers for soil stabilization and fertility and preventing soil erosion and desertification. Like most legumes, chickpea performs BNF with compatible root-nodule bacteria and increases the combined N input to soil. More than 70% of the nitrogen economy of chickpea is obtained through symbiosis with nodulating bacteria, giving chickpea rhizobia a great agricultural value. Moreover, inoculation of selected rhizobia inoculants on chickpea has beneficial effects on yield (Ogola and John, 2015). In Turkey, it contributes to about 18% of the protein consumption. In 1984, Turkey produced more than one-third of the world's chickpea. However, Turkey's production of chickpea is in decline for both economical and phytopathological problems (Aybegun *et al.*, 2014). Fusarium wilt and Ascochyta blight diseases are the main biotic stress factors negatively affecting chickpea yield in Turkey and throughout the world, causing up to 100% yield losses. Since most Turkish soils are nitrogen deficient, rhizobia could increase chickpea yield at low cost and preserve water resources from pollution by nitrates. Additionally, rhizobia can enhance growth by excreting growth-promoting factors (Singh and Singh, 2018) and increase the plant resistance against soil-borne fungi (Suman and Yadav, 2015). To improve chickpea inoculation effect, characteristics of indigenous rhizobia populations must be determined. To our knowledge, this is the first study addressing the characterization of chickpea rhizobia covering wide areas in Turkey. In this study, Isolates with promising features can be used as inoculants to increase chickpea yield and decrease dependence on synthetic fertilizers.

MATERIALS AND METHODS

Plant collections and isolations of rhizobia: Nodulated plants were collected between May and July of the year 2015 from farmer's fields in 14 provinces in Turkey (Table 1). Samples were transported in sterile plastic bags to the Mycology laboratory, Gaziantep University, Turkey, where the study was conducted. Nodules were surface sterilized, crushed and streaked onto yeast extract mannitol agar containing 0.0025% (w v^{-1}) Congo red (CRYEMA) and incubated at 28±2°C for 3-5 days.

Morphological characterizations: Colonies were characterized on CRYEMA based on their color, shape, diameter, elevation, margin and gum production.

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Table 1: Chickpea rhizobia used in this study and their collection sites.

Origin	GPS attributes	Isolates
Adana	N[36º05'24"] E[37º18'41"]	Ada2/Ada5A/Ada6A/Ada8/Ada9/Ada10
Adiyaman	N[37º35'02"] E[37º 58' 57"]	Adi1/Adi4/Adi5/Adi6/Adi7/Adi9/Adi12A/Adi14/Adi15/Adi16A1/ Adi17
Afyon	N[38º25'43"] E[30º 38' 18"]	Afy1/Afy3/Afy5
Aksaray	N[38º31'43"] E[34º24'42"]	Aks1/Aks2/Aks3A/Aks3B/Aks4A/Aks5/Aks7/Aks10
Amasya	N[40º34'47"] E[36º19' 06"]	Amas3/Amas4/Amas7
Corum	N[40º21'34"] E [34º51'14"]	Cor2A/Cor3/Cor4/Cor5/Cor7/Cor9
Eskisehir	N[39º30'03"] E[30º45'13"]	Esk6/Esk7A/Esk7B
Gaziantep	N[37º16'11"] E[37º24'03"]	Gbak/Gbur1A/Gbur1B/Gbur3/Gogu1/Gogu2/Gsar1A/Gsehinb/Gsehit1 Gsenlik1/Gsut2/Gyav1/Gyav3A/Gyes1
Hatay	N[36º28'43"] E[36º16'36"]	Hat1/ Hat2/ Hat3/ Hat4A
Kahraman	N[38º22'54"] E[36º54'34"]	Kah1/Kah2A/Kah3/Kah6/Kah7/Kah8/Kah9/Kah10/Kah13
Kirsehir	N[39º19'27"] E[33º57'16"]	Kir1/Kir4/Kir5A/Kir9/Kir10/Kir12/Kir15/Kir17A/Kir19/Kir20/Kir21
Konya	N[37º43'51"] E[31º46'32"]	Kon2/Kon3/Kon7/Kon9
Kutahya	N[39º16'11"] E[30º10'40"]	Kut2/Kut9
Mersin	N[36º29'42"] E[33º58'06"]	Mer1/Mer2/Mer3/Mer4/Mer5A/Mer5B/Mer6A/Mer6B/ Mer7/ Mer8/Mer9/Mer10A/Mer11/Mer12/Mer13/Mer16/Mer17/Mer18/Mer26
Tokat	N[40º15'32"] E[35 46'58"]	Tok1/Tok2/Tok3/Tok4
Urfa	N[37º03'13"] E[38º11'42"]	Urfa2
Usak	N[38º31'20"] E[29º26' 44"]	Usak1/Usak2/Usak13/Usak17
Yozgat	N[39º51'13"] E[34º57'19"]	Yoz2/Yoz6/Yoz7/Yoz8/Yoz11/Yoz14/Yoz15/Yoz16

Appearance under microscope and Gram staining were done for more specific identification. Isolates were tested on YEMA containing 0.025% bromothymol blue (BTB) for acid production. To confirm them as rhizobia, isolates were streaked on glucose peptone agar (GPA) with BTB indicator and on lactose agar medium (Vincent 1970).

Nodulation tests: Sterilized seedlings were inoculated by bacterial broth in modified Leonard jars. Three replicates were used for each isolate and un-inoculated plants were used as negative controls. Plants were fertilized with a nitrogen-free nutrient solution once a week and received water every three days. The existence of nodules was recorded for 30 days after planting.

Phenotypic and biochemical characterizations

Utilizations of C- and N- sources: Carbon sources were prepared as 10% (w v^{-1}) solutions in sterilized water and added to YEMA modified by reducing its yeast extract to 0.05 g l^{-1} to a final concentration of 1% (w v^{-1}). The nitrogen sources were added to a final concentration of 0.5 g L^{-1} to the basal medium (Fig 2).

NaCl, pH and temperature tolerance: Isolates were inoculated on YEMA and values of pH, NaCl (w v^{-1}) and incubation temperatures were adjusted (Fig 3).

Intrinsic antibiotic and heavy metal resistance: This was tested on YEMA containing concentrations (µg ml^{-1}) of the following filter sterilized antibiotics or heavy metals (Fig 4): Nalidixic acid (NA), streptomycin (Str), kanamycin (KA), erythromycin (Ery), Ampicillin (Amp), tetracycline (tetr), $CuSO_4$ (Cu), HgCl2 (Hg), NiCl2, 6H2O (Ni), $ZnSO_4$, $7H_2O$ (Zn), CdCl2 (Cd) and K2Cr2O7 (Cr).

Biochemical tests: Catalase, oxidase, amylase, urease, gelatinase, indole acetic acid (IAA) production and methyl red (MR) activities were tested (Holt *et al*., 1994).

Statistical analysis: The final matrix contained 120 isolates and 71 traits. Results were coded 1 for positive and 0 for

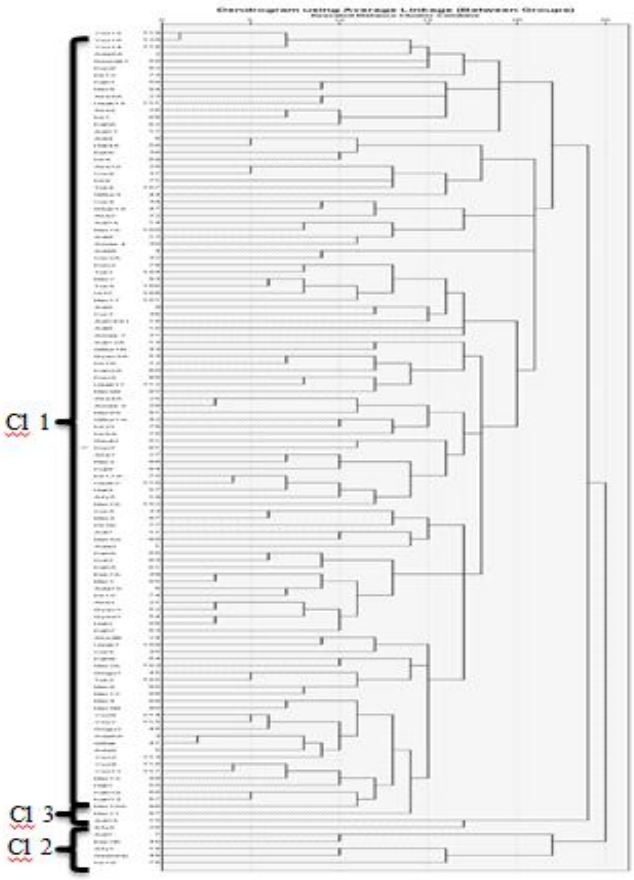

Fig 1: Phenogram showing clusters (Cl) of 120 chickpea isolates from different areas of Turkey.

negative and hierarchical clustering analysis (Fig 1 and Table 2) was carried out using IBM SPSS version 23 software. Pearson correlation interval measure and between-groups linkage method were used for the hierarchical clustering.

RESULTS AND DISCUSSION

Results revealed the diversity of chickpea-nodulating rhizobia in Turkey. Such a heterogeneity was reported before (Kucuk and Kivanc, 2008; Jida and Assefa, 2012). Cluster analysis placed isolates into 3 clusters at 25% similarity (Table 2). Cluster I was the largest with 113 strains. They came from different origins and metabolized a wide range of carbon and nitrogen substrates. They moderately tolerated 1 to 3% NaCl and pHs between 5 and 10 and were sensitive to temperatures above 35°C. They showed high tolerance to Cr, Cd, Zn, Ni, NA, and KA and were IAA and urease positive. Cluster I isolates showed a close relationship with *Mesorhizobium ciceri*, based on the description of Jarvis *et al*. (1982) and Nour *et al*. (1994, 1995). Cluster 2 had 5 isolates *viz*. Adi1, Afy1, Esk7B, Gsehinb, and Kir19. They displayed higher tolerance to salinity and temperature than isolates in cluster I as they all grew at 3% Na and between 35-45°C. They also tolerated all concentrations of Zn, tetracycline, and streptomycin and were all urease positive. Based on these traits and description of Jarvis *et al*. (1982) and Nour *et al*. (1994, 1995) they showed a close relationship with *Mesorhizobium mediterraneum*. Two isolates namely Adi15 and Afyon5 fell into cluster 3. Unlike other isolates, cluster 3 isolates didn't utilize starch as a sole carbon source, but grew well with sorbitol and citrate and endured salinity up to 4%. They all grew between 20-40°C and withstand all

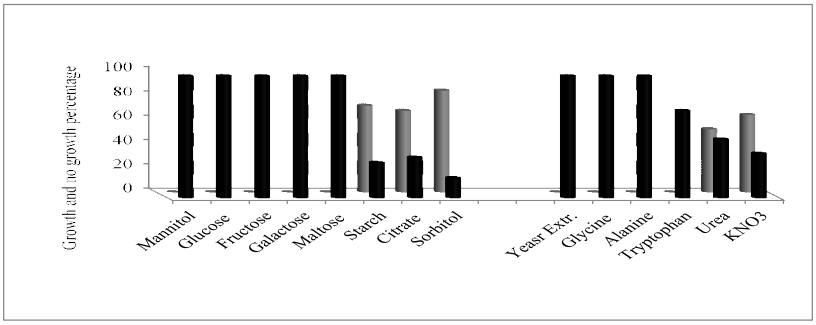

Fig 2: Percentage of growth with different carbon and nitrogen sources. Black columns indicate growth while grey indicate no growth percentage of isolates.

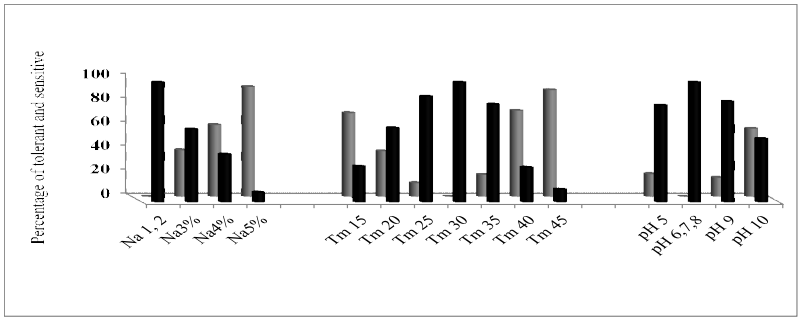

Fig 3: Percentage of tolerant isolates at different values of salinity (Na), temperature (Tm) and pH. Different values of the same variable type gave the same result were combined together under one column. Black columns indicate tolerant while grey indicate sensitive.

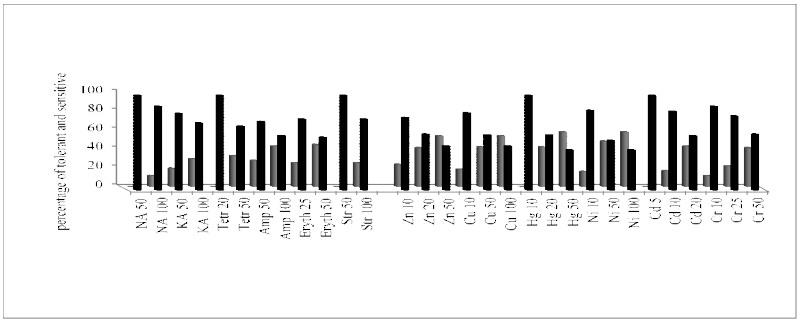

Fig 4: Percentage of tolerant and sensitive isolates at different concentrations (µg ml^{-1}) of antibiotics and heavy metals. Black columns indicate tolerant while grey indicate sensitive.

Table 2: Clustering of phenotypic traits of 120 chickpea isolates.

Characteristics	**Cluster1 (n=113*)**	**Cluster2 (n=5)**	**Cluster3 (n=2)**
Cofirmation tests			
GPA,Ketolactose	4,7	5,1	0,1
Carbon utilization			
Starch,Sorbitol,Citrate	33,22,46	2,2,2	0,2,2
Nitrogen utilization			
Tryptophan,Urea,KNO3	83,56,41	2,1,2	1,1,1
Salinity tolerance			
3,4,5%	67,43,8	5,3,1	2,2,1
Temperature Tolerance			
15,20,25,35,40,45^{o} C	34,81,102,91,28,8	1,2,2,5,5,5	1,2,2,2,2,0
pH tolerance			
5,9,10	92,100,61	4,3,3	1,2,0
Heavy metal resistance			
Zn 10,20,50	86,66,51	5,5,5	1,0,0
Cu 10,50,100	94,68,55	2,1,1	2,1,0
Hg 20,50	64,47	4,2	2,1
Ni 10,50,100	94,58,47	5,3,2	2,2,2
Cd 10,20	97,67	1,0	2,2
Cr 10,25,50	109,89,67	5,4,3	2,1,1
Antibiotic resistance			
NA 100	102	2	2
KA 50,100	92,82	4,3	1,0
Tetr 50	75	5	1
Amp 50,100	84,66	3,3	0,0
Eryth 25,50	86,66	2,0	2,1
Strept 100	83	5	2
Enzyme activities			
Urease	67	5	0
IAA	23	1	2

* N is the total number of isolates per cluster and the column numbers are the isolates giving a positive reaction. Commas (,) were used to separate different values of the same trait and their corresponding responses in clusters in a respective series. Phenotypes that gave the same results in all strains were omitted from the table.

concentrations of Ni, Cd, NA, and streptomycin, but were sensitive to Ampicillin. Based on these traits and description of Jarvis *et al*. (1982) and Nour *et al*. (1994, 1995), they showed a close relationship with *Mesorhizobium* sp. clustering of isolates didn't correlate with their collection sites. Atypical example of this was isolates Afy1 and Kir19 which came from diverse sites but clustered together in cluster 2. The majority of isolates produced mucously, circular, smooth-margined, and watery to creamy colonies with 2–4 mm diameter after 1-3 days growth on CRYMA. Nour *et al*. (1995) reported similar characteristics for chickpea Mesorhizobia. Rai *et al.* (2013) and Gauri *et al.* (2012) also characterized chickpea Mesorhizobia on the basis of their colony shape, colour, and texture. Our isolates were all Gram-negative rods, they all re-nodulated their host plants, didn't absorb CR in YEMA and didn't grow with GPA or ketolactose which are all distinctive features of rhizobia. However, 9 isolates showed unexpected growth with GPA and positive results with ketolactose. All isolates changed the color of YEMA supplemented with BTB to yellow indicating they were acid producers/fast growers (Datta *et al.,* 2015). They showed variable growth with starch, sorbitol, and citrate while utilized the other sources of carbon. Utilization of different compounds as sole carbon and nitrogen sources is a useful trait for isolates differentiation (Hungria *et al.*, 2001). Chickpea Mesorhizobia were reported to utilize different carbon sources (L'taief *et al.,* 2007) and the variable types of utilized carbohydrates can be used as a diagnostic feature (Kucuk and Kivanc, 2008). Utilization of different carbon sources by our fast growing isolates cope with findings of Stowers (1985). Unlike the findings of Kucuk and Kivanc (2008), some of our isolates utilized starch and citrate. De Oliveria *et al.* (2007) observed rhizobia from different sources utilized starch. Also, citrate utilization by chickpea Mesorhizobia and rhizobia from other legumes was also reported (Wani and Khan 2013; Datta *et al.,* 2015). Similar to previous studies (Kucuk and Kivanc, 2008), our isolates utilized different nitrogen sources which is a desirable characteristic for field studies and gives ecological competences in the soil (Jida and Assefa, 2012). Regarding salinity stress, all isolates tolerated 1 and 2% salt in line with findings on chickpea Mesorhizobia (L'taief *et al.,* 2007). Rabie and Alamadini (2005) reported growth of rhizobia was not affected by low and moderate levels of salinity. Moreover, 10 isolates *viz.* Ada10, Adi4, Adi12A, Adi16A, Amas7, Cor7, Tok1 and Tok4 (cluster 1), Esk7B (cluster 2) and Afy5 (cluster 3) grew at5% NaCl. Highly saline-tolerant *Mesorhizobium ciceri* had also been reported (Soussi *et al.,* 2001; Singh *et al.,* 2015). Tolerance of our isolates to high salinity confirmed the conclusion that fast-growing rhizobia are salt tolerant (El Sheikh and Wood, 1989). Kucuk *et al.* (2006) demonstrated rhizobia strains grew variably at 5% NaCl. Despite originating from the same site, isolates Adi12A and Adi14 had different maximum tolerance at 1 and 5% salt, respectively. Similarly, Maatallah *et al.* (2002) observed variations in salt tolerance with chickpea Mesorhizobia from the same site. The majority of our isolates grew at 20-35°C. Maatallah *et al*. (2002) described a similar maximum temperature growth at 20–35°C for chickpea Mesorhizobia. The maximum growth temperature for chickpea rhizobia was reported to be 40°C, both for *M. ciceri* and *M. mediterraneum* (Nour *et al*., 1994, 1995). Isolates in cluster 2 showed thermotolerant features as they had optimum temperature between 35-45°C. Similarly, Soussi *et al.* (2001) observed thermotolerant chickpea Mesorhizobia. Some isolates grew at 15°C in tune with Rai *et al.* (2012) who reported growth of chickpea Mesorhizobia at 15°C and 42°C. Deora and Singhal (2010) demonstrated a slight variation in the medium pH might affect the growth of rhizobia. 80% of our isolates grew at pH 5 and they all grew at pH 6-8. Similar findings reported chickpea Mesorhizobia grew at pH 5-8 (Maatallah *et al.*,2002; L'taief *et al.*,2007). However, 84 and 53% of our isolates tolerated pH 9 and 10, respectively, in line with Nour *et al*. (1994) who observed chickpea *Mesorhizobium ciceri* were more tolerant than rhizobia with a pH tolerance range of 4.5–10. Moreover, Singh *et al*. (2015) reported alkalotolerant chickpea Mesorhizobia that grew well at pH 10. Icgen *et al*. (2002) showed chickpea rhizobia displayed a tendency to neutralize the pH of the medium when grown freely in media with different pH values, which explains the success of growth in both acidic and alkaline conditions. Jordan (1984) reported fast-growing strains were more sensitive to antibiotics. Conversely, our fast-growing isolates were insensitive to most of the assessed antibiotics (Fig 4). The highest tolerance was in the order, NA> Str > KA > Tetr > Amp > Ery. Wani *et al.* (2013) reported similar results for chickpea Mesorhizobia. The AR pattern had been used to identify diversity among strains of rhizobia (Jida and Assefa, 2012). As reported for chickpea Mesorhizobia (Wani, *et al.,* 2013), our isolates were tolerant to Hg, Cr, and Cu which could be used as selective agents for isolates (Kucuk and Kivanc, 2008). For biochemical activities, our isolates were all catalase and oxidase positive and MR and amylase negative which correlates with previous reports (Singh *et al.,* 2013). Similar to our findings, Gauri *et al.* (2012) reported positive urease and IAA among rhizobia strains. Hunter (2007) reported negative gelatinase activity was a feature of Rhizobium which in line with our observations.

CONCLUSION

We conclude that rhizobia strains isolated from chickpea nodules in Turkey are phenotypically diverse. Based on their morphological, confirmatory, physiological and biochemical characteristics, most of the isolates belong to the *Mesorhizobium* genus and are closely related to *Mesorhizobium ciceri* and *Mesorhizobium mediterraneum*. This study should be followed by molecular and sequencing approach to evaluate the genotypic diversity and identify the isolates more precisely to the species level.

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