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Nitrogen fixation capacity of *Azotobacter* spp. strains isolated from soils in different ecosystems and relationship between them and the microbiological properties of soils

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Abstract: *The objectives of this study were to count and culture Azotobacter spp. in sampled soils, to determine the nitrogen (N) fixing capacity by Azotobacter spp. in pure culture and different soils, and to explore the relationships between N fixation capacity of Azotobacter spp. and microbiological properties of soils in Northern Anatolia, Turkey. Statistically significant relationships were found between the population of Azotobacter spp. in soils and microbial biomass C (C_{mic}), dehydrogenase (DHA), β-glucosidase (GA), alkaline phosphatase (APA) and arylsulphatase (ASA) activities. However, relationships between the population of Azotobacter spp. and basal soil respiration (BSR), urease (UA) and catalase (CA) activities were insignificant. The N fixation capacities of native 3 day old Azotobacter chroococcum strains added to Ashby Media varied from 3.50 to 29.35 μg N ml^{-1} on average 10.24. In addition, N fixation capacities of Azotobacter spp. strains inoculated with clayey soil, loam soil, and sandy clay loam soil during eight week incubation period were 4.78-15.91 μg N g^{-1}, 9.03-13.47 μg N g^{-1} and 6.51-16.60 μg N g^{-1}, respectively. It was concluded that the most N fixation by Azotobacter spp. was in sandy clay loam soils.*

Key words: *Azotobacter spp., Soil, Texture, N fixation, Microbiological properties*
PDF of full length paper is available with author ***(*ridvank@omu.edu.tr)***

Introduction

After photosynthesis, the second most important biological process on earth is biological nitrogen fixation, which is the reduction of atmospheric nitrogen (N_2) to two molecules of ammonia. Nitrogen fixation is mediated exclusively by prokaryotes, including many genera of bacteria, cyanobacteria, and the actinomycete *Frankia* (Alexander, 1977; Ravikumar *et al.,* 2007). Nitrogen-fixing microbes can exist as independent, free-living organisms or in associations of differing degrees of complexity with other microbes and plants (Sylvia *et al.,* 2005). Nitrogen, the most abundant element in the atmosphere is the limiting element for the growth of most organisms (Davey and Wollum, 1984). Constraints that soil places on microbial biota, in terms of nutritional limitations and environmental stresses, it is not surprising that many soil organisms interact with each other to overcome some of these limitations (Alexander, 1977; Chandra *et al.,* 2004; Sylvia *et al.,* 2005).

Azotobacter spp. are free-living aerobic bacteria dominantly found in soils. They are non-symbiotic heterotrophic bacteria capable of fixing an average 20 kg N/ha/per year. Besides, it also produces growth promoting substances and are shown to be antagonistic to pathogens. *Azotobacter* spp. are found in the soil and rhizosphere of many plants and their population ranges from negligible to 10^4 g^{-1} of soil depending upon the physico-chemical and microbiological (microbial interactions) properties. *Azotobacter chroococcum* is the most prevalent species found but other species described include *A.agilis, A.vinelandii, A. beijerinckii, A.insignis, A.macrocytogenes* and *A.paspali* (FAO, 1982). In soils, *Azotobacter* spp. populations are affected by soil physico-chemical (*eg.* organic matter, pH, temperature, soil depth, soil moisture) and microbiological (*eg.* microbial interactions) properties. As far as physico-chemical soil properties are concerned, numerous studies have focused on the nutrients (*i.e.* P, K, Ca) and organic matter content and their positive impact on *Azotobacter* spp. populations in soils (Pramanix and Misra, 1955; Bescking, 1961; Jensen, 1965; Burris, 1969). In contrast, little information is available on the relationships among *Azotobacter* spp. populations, their activities, and microbiological properties of soils such as microbial biomass C, basal soil respiration, and enzyme activities (dehydrogenase, catalase, glucosidase, urease, phosphatase and sulphatase). Since soil biological properties are indicators for soil quality, soil health and fertility, examining the relationships between these parameters and *Azotobacter* spp. populations have vital role for agricultural practices and management application.

The objectives of this study were to (1) count and culture *Azotobacter* spp. in sampled soils, (2) determine the nitrogen fixing capacity by *Azotobacter* spp. in pure culture and different soils, and (3) explore the relationships between N fixation capacity of *Azotobacter* spp. and microbiological properties (dehydrogenase, catalase, β-glucosidase, urease, alkaline phosphatase and arylsulphatase) of soils in Northern Anatolia, Turkey.

Materials and Methods

Soil collection: Soil samples were taken from widely different geographic, geologic, and climatic conditions in Northern Anatolia, Turkey (Fig. 1). Since large quantities of soil were needed, the exposed surface was scraped off and the soil was taken from a

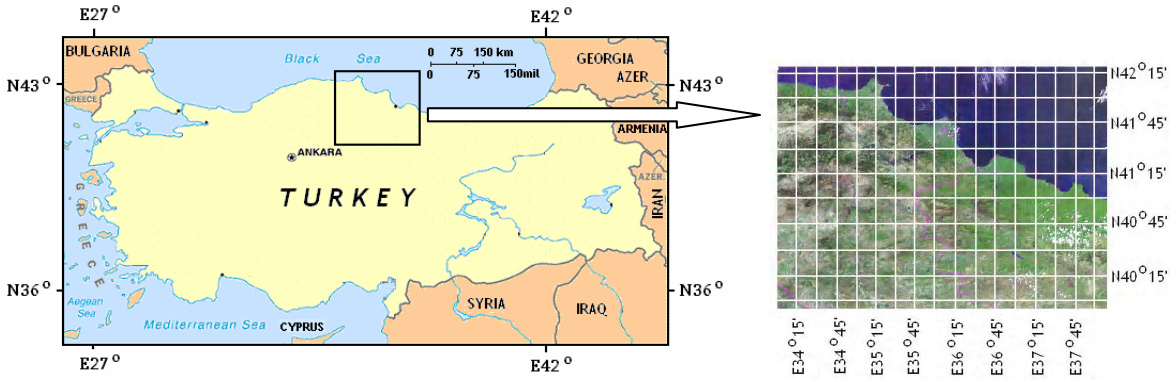

Fig. 1: Topographic map of study area showing the location of Northern Anatolia, Turkey

depth of approximately twenty centimeters. Soil samples weighing approximately 500 g were taken by means of a sterile soil corer, in May 2005. The corer was sterilized with 95% ethanol before each use. The entire volume of soil was passed through a coarse sieve (<2 mm) to pulverize, remove any foreign material, and thoroughly mix it, and the desired quantity removed to sterile bottles. These samples were transported to the laboratory at 4°C and they were used to examine microbiological (enzyme activities, microbial biomass and respiration) properties of soils and culturing *Azotobacter* spp. at the field moisture condition. Each sample was stored in sterile bottles at 4°C in the refrigerator for no longer than the time required for analysis. Additionally, secondary soil samples were collected for exploring of physico-chemical properties of soils.

Physico-chemical properties of soils: Physico-chemical analyses was conducted on air-dried samples from which crop residues, root fragments and rocks larger than 2 mm were removed and stored at room temperature. Selected physico-chemical properties were explored by the following methods: soil particle size distribution by the hydrometer method (Bouyoucos, 1951), pH in 1:1 (w/v) in soil: water suspension by pH - meter (Rowell, 1996), electrical conductivity (EC) in 1:1 (w/v) in soil: water suspension by EC - meter (Rowell, 1996), $CaCO_3$ content by volumetric method (Martin and Reeve, 1955), total nitrogen by the Kjeldahl method (Bremner, 1965), available P by the 0,5 M $NaHCO_3$ extraction (Olsen and Sommers, 1982) and exchangeable cations (K, Ca and Mg) by the 1N NH_4OAc extraction (Rowell, 1996). All soil samples were sieved through a 150 μm opening sizes to measure the total organic matter content by the wet oxidation method with $K_2Cr_2O_7$ (Walkey, 1946). The available micronutrient (Fe, Cu, Zn and Mn) content of the soils was determined by the extraction with 0.005 M DTPA + 0.01 M $CaCl_2$ + 0.1 M TEA and analyzed by atomic absorption spectrophotometer (Lindsay and Norvell, 1978).

Soil microbiological properties: Microbiological properties were studied for the field-moist soil. The soil moisture content was measured after drying at 105 C for 24 hr. All results on microbiological properties were expressed on the basis of moisture-free weights.

Microbial biomass and basal soil respiration: Microbial biomass carbon (C_{mic}) was determined by using the substrate-induced respiration method by Anderson and Domsch (1978). A moist soil equivalent to 10 g oven-dry soil was amended with a powder mixture containing 40 mg glucose. The CO_2 production rate was measured hourly using the method described by Anderson (1982). The pattern of respiratory response was recorded for 4 hr. Microbial biomass carbon (C_{mic}) was calculated from the maximum initial respiratory response in terms of mg C g^{-1} soil as 40.04 mg CO_2 g^{-1} + 3,75. Data were expressed as μg C g^{-1} dry soil.

Basal soil respiration (BSR) at field capacity (CO_2 production at 25°C without addition of glucose) was measured according to Anderson (1982); by alkali ($Ba(OH)_2.8H_2O$ + $BaCl_2$) absorption of the CO_2 produced during the 24hr incubation period, followed by titration of the residual OH^- with standardized hydrochloric acid, after adding three drops of phenolphthalein as an indicator. Data were expressed as μg CO_2-C g^{-1} dry soil.

Soil enzyme activities: Dehydrogenase activity (DHA) was explored according to Pepper *et al.* (1995). One ml of 3% TTC (2,3,5-triphenyltetrazoliumchlorid) solution and 2.5 ml pure water were added to 6 g of soil 30 mg glucose and the samples were incubated for 24 hr at 25°C. The formation of TPF (1,3,5 triphenylformazan) was found spectrophotometrically at 485 nm and results were expressed as μg TPF g^{-1} dry soil.

Catalase activity (CA) was measured by the method of Beck (1971). Ten ml of phosphate buffer (pH, 7) and 5 ml of a 3% H_2O_2 substrate solution were added to 5 g of soil. The volume (ml) of O_2 released within 3 minutes at 25°C was measured. Three replicates of each soil were tested and controls were tested in the same way, but with the addition of 2 ml of 6.5% (w/v) NaN_3. Results were expressed as ml O_2 g^{-1} dry soil.

β-glucosidase activity (GA) was measured according to Eivazi and Tabatabai (1988). 0.25 ml toluene, 4 ml TRIS (hydroximethyl) aminomethane buffer (pH, 12) and 1 ml of 0.05 M *p*-nitrophenyl -D-glucopyranoside solution were added to the 1 g soil and the soil were incubated for 1 hr at 37°C. The formation

of *p*-nitrophenol (*p*-NP) was determined spectrophotometrically 410 nm and results were expressed as μg *p*-NP g^{-1} dry soil.

Urease activity (UA) was measured by the method of Hoffmann and Teicher (1961). 0.25 ml toluene, 0.75 ml citrate buffer (pH, 6.7) and 1 ml of 10% urea substrate solution were added to the 1 g soil and the soils were incubated for 3 hr at 37°C. The formation of ammonium was found out spectrophotometrically at 578 nm and results were expressed as μg N g^{-1} dry soil.

Alkaline phosphatase activity (APA) was measured according to Tabatabai and Bremner (1969). 0.25 ml toluene, 4 ml phosphate buffer (pH, 8.0) and 1 ml of 0,115 M *p*-nitrophenyl phosphate (disodium salt hexahydrate) solution were added to the 1 g sample and the samples were incubated for 1 hr at 37°C. The formation of *p*-nitrophenol (*p*-NP) was explored spectrophotometrically at 410 nm and results were expressed as μg *p*-NP g^{-1} dry sample.

Arylsulphatase activity (ASA) was measured according to Tabatabai and Bremner (1970). 0.25 ml toluene, 4 ml acetate buffer (pH, 5.5) and 1 ml of 0.115 M *p*-nitrophenyl sulphate (potassium salt) solution were added to the 1 g sample and the samples were incubated for 1 hr at 37°C. The formation of *p*-nitrophenol (*p*-NP) was determined spectrophotometrically 410 nm and results were expressed as μg *p*-NP g^{-1} dry sample.

Counting and culturing *Azotobacter* spp.: Soil samples, 10 g in weight, were shaken with 90 ml of sterile distilled water at 30°C for 15 min. After this, solutions were serially diluted in a proportion of 1:10 up to 10^9in sterile distilled water. From these, 1 ml of each dilution was planted on to triplicate agar Ashby medium (5g glucose, 5g mannitol, 0.1 g $CaCl_2.2H_2O$, 0.1 gr $MgSO_4.7H_2O$, 5 mg $Na_2MoO_4.2H_2O$, 0.9 g K_2HPO_4, 0.1 g KH_2PO_4, 0.01 g $FeSO_4.7H_2O$, 5g $CaCO_3$ and 15 g agar in 1 L distilled water, pH 7.3) and incubated at 30°C for 72 hour. To verify the purity of the isolates, a Gram strain was performed. Identification of isolated *Azotobacter* spp. colonies on Ashby medium was based on morphological, biochemical and culture methods. *Azotobacter* cell numbers were counted using plate-count method according to Clark (1965).

Determination of nitrogen fixing capacity by *Azotobacter* spp. in pure culture: *Azotobacter* spp. isolates were grown in 10 ml Ashby medium (without agar) in 20 ml test tube on a rotary shaker (125 rpm) under continuous airflow at 30°C for 72 hr. Cell concentrations were determined as 10^5 CFU ml^{-1} of each isolate by plate counts on agar Ashby medium. The non-inoculated media served as control. Afterwards, the concentration of nitrogen in each liquid culture was measured by digestion and subsequent measurement by the Kjeldahl method (Bremner, 1965). The quantities of nitrogen reported represent the average of duplicate cultures after deducting the average of duplicate controls.

Determination of nitrogen fixing capacity by *A. chroococcum* in soil: Soil samples consisting of three different textural groups, the properties of which are given in Table 1, were air-dried in a laboratory and sieved through 2 mm screens. Non-sterilized samples (250 g air-dried soil) were placed in 300 ml cylindrical plastic containers. Ten ml of 72 hr old *Azotobacter* spp. isolates (fifty five total isolates, 10^9 CFU ml^{-1} of each isolate) in Ashby medium (without agar) were prepared as pure cultures, and added to the containers. These were kept under continuous airflow and were incubated in the laboratory at 25°C for 8 weeks. The moisture content of all soil incubated in the laboratory was adjusted up to approximately the optimum level for aerobic bacterial activity. The samples were first adjusted to 50% of the soil water holding capacity by adding sterile distilled water. The non-inoculated soil was used as a control. The soil moisture was kept at the same level (50% water holding capacity (WHC) by adding sterile distilled water at regular intervals throughout the incubation period. There were three replicates per treatment. At the end of the incubation period, the contents of N in each soil were quantified by digestion and subsequent measurement by the Kjeldahl method (Bremner, 1965).

Statistical analysis: All measurements were performed in triplicate, and all values reported are mean of triplicate determinations. Pearson correlation coefficients and *p*-values were calculated for all possible variable pairs and were performed by using SPSS 11.0. The asterisks * and ** indicate significance at $p<0.05$ and 0.01 respectively.

Results and Discussion

Soil physico-chemical and microbiological properties: Physico-chemical properties of soils are given in Table 2. The results showed that soil samples have slightly alkaline (7.3-7.8) in pH, adequate (>3%) in organic matter content, moderate (5–15%) in $CaCO_3$ content, high (>0.25%) in nitrogen, low (< 8 μg g^{-1}) in phosphorus, adequate (>1.5 meq 100 g^{-1}) in potassium, adequate (>5 meq 100 g^{-1}) in calcium, adequate (>2.5 meq 100 g^{-1}) in magnesium, adequate (>4.5 μg g^{-1}) in iron, adequate (> 0.2 μg g^{-1}) in copper, adequate (>1.0 μg g^{-1}) in zinc and adequate (>2.0 μg g^{-1}) in manganese generally. Research results also showed that soil particle size distribution has been changed in widely range. Soils can be classified as clayey (14 samples), sandy loam (13 samples), clay loam (11 samples), sandy clay loam (7 samples) and loam (5 samples) based on to its texture. Descriptive information for sampled locations in northern Anatolia, Turkey and microbiological properties, and number of *Azotobacter* spp. sampled soils are given in Table 3. The N fixing capacity in different 3 soils by *A.chroococcum* are given in Table 4.

Relationships between *Azotobacter spp.* population and microbiological properties in sampled soils: The study sites had different ecological conditions with quite different vegetation, agricultural practices, pH and $CaCO_3$ contents. For this reason, among 22 soils, some didn't have *Azotobacter* spp., while some did. There have been numerous studies about the effects of physico-chemical properties on *Azotobacter* spp. and their activities such as population and N fixation in association with the physico-chemical properties of the soil. Our findings confirmed with the previous

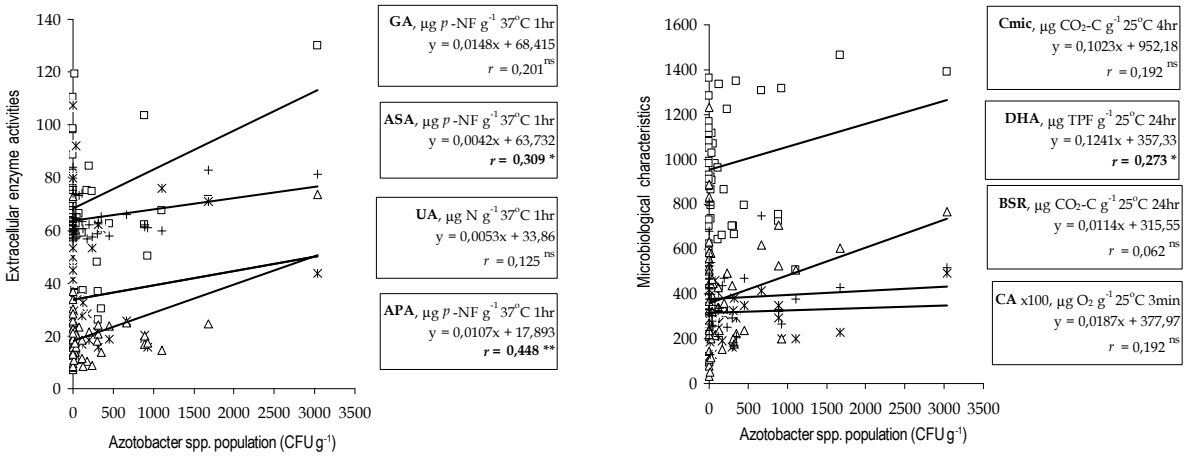

Fig. 2: The relationships between soil microbiological properties and *Azotobacter* spp. cell number in all sampled soils (n=50)

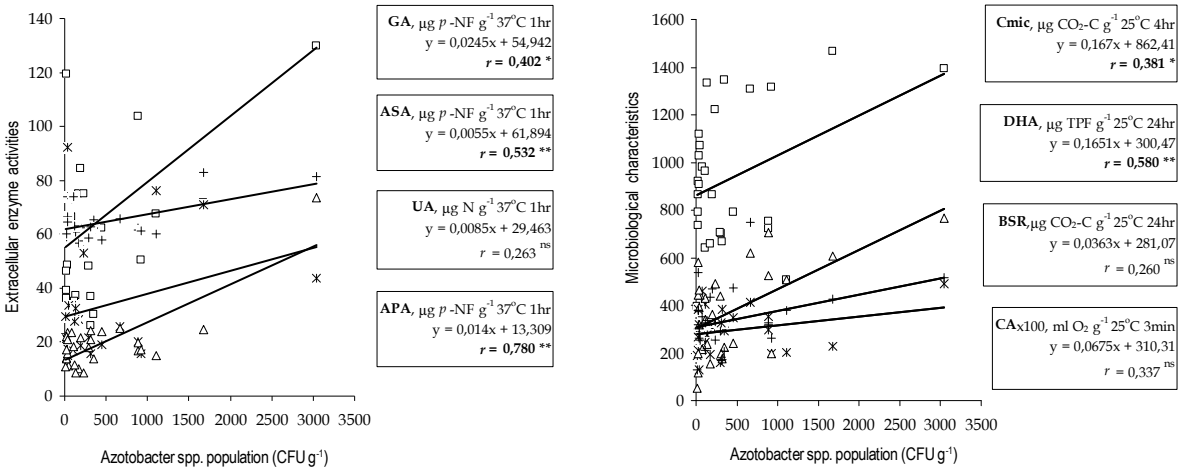

Fig. 3: The relationships between soil microbiological properties and *Azotobacter* spp. cell number in *Azotobacter* spp. included soils (n=28)

research results on relationships between *Azotobacter* spp. and physico-chemical properties. The extent of N fixation by *Azotobacter* spp. and its population is explored by using soil-related factors. Oxygen supply has considerable importance: cell-free extracts of *Azotobacter* spp. only fix nitrogen under aerobic conditions (Stewart, 1966). Among the climatic factors, the soil moisture content, which may be seasonally deficient and N fixation may reduce growth of a cell. Under normal field conditions the temperature seldom acts as a limiting factor. Optima for growth are 29-37°C for *Azotobacter* spp. (Dobereiner, 1969). Nitrogen fixation by free living organisms is a process that needs considerable amounts of organic matter to be effective. According to Dobereiner (1969) free living organisms would be able to fix between 12 and 30 mg N g^{-1} of carbon source. In the absence of nitrogen in the soil, for ten kilograms of N fixed ha^{-1}, the bacteria would have used between 330 and 830 kg of carbon, taking into account that all other conditions are optimized.

In this study, it was fixed based on the research results that there were the positive relationships between *Azotobacter* spp. population in soil and microbiological properties (Fig. 2, 3). In addition, it was clear that the results obtained from soils (n=28) containing only *Azotobacter* spp. significantly differed from the results, obtained from soils not containing *Azotobacter* spp. However, correlation coefficients obtained from statistical evaluation in soils containing *Azotobacter* spp. was found to be greater and statistically significant.

Microbiological properties of soil may show considerable differences associated with the sensitivity to *Azotobacter* spp. populations (Kisten *et al.,* 2006; Bakulin *et al.,* 2007). In this study, with the exception of UA, BSR and CA, generally significant positive relationships were observed between the *Azotobacter* spp. population in soil and microbiological properties (Fig. 2,3).

We accept that C_{mic} gives an idea of the potential microbial activity of a soil (Nannipieri *et al.,* 1990). Similarly, Anderson (1982)

Table - 1: Physico-chemical properties of the soils used in the experiment

Soil physico-chemical properties	Experimental soil 1	Experimental soil 2	Experimental soil 3
Sand (%)	70.53	28.74	37.81
Silt (%)	6.80	16.20	38.60
Clay (%)	22.67	55.06	23.59
Texture class	Sandy clay loam (SCL)	Clay (C)	Loam (L)
pH (1:1)	7.45	8.23	7.40
EC (dSm^{-1})	0.79	0.54	0.45
$CaCO_3$ (%)	6.22	5.25	14.15
Organic matter (%)	2.94	2.90	2.46
Total N (%)	0.25	0.16	0.10
C:N ratio	6.83	10.51	14.27
Available P ($\mu g\ g^{-1}$)	7.77	8.41	2.81
Exchangeable K (meq $100\ g^{-1}$)	0.94	2.00	1.08
Exchangeable Ca (meq $100\ g^{-1}$)	9.00	8.42	8.71
Exchangeable Mg (meq $100\ g^{-1}$)	3.04	1.29	3.79
DTPA- Fe ($\mu g\ g^{-1}$)	4.33	1.42	10.01
DTPA- Zn ($\mu g\ g^{-1}$)	0.18	0.03	0.28
DTPA- Cu ($\mu g\ g^{-1}$)	0.79	1.66	0.78
DTPA - Mn ($\mu g\ g^{-1}$)	5.02	3.30	4.12

Table - 2: Range, mean and standard deviations of physico-chemical properties in the studied soils (n=50)

Soil physico-chemical properties	Range	Mean	Standard deviation
Sand (%)	18.96 - 73.59	42.95	14.56
Silt (%)	6.80 - 40.46	26.38	6.73
Clay (%)	11.02 - 58.25	30.68	13.22
pH (1:1)	4.85 - 8.25	7.38	0.75
EC (dSm^{-1})	0.15 - 2.28	0.58	0.45
$CaCO_3$ (%)	< 0.01 - 21.52	7.70	5.73
Organic matter (%)	1.76 - 17.92	3.78	2.63
Total N (%)	0.08 - 0.98	0.33	0.19
C:N ratio	1.73 - 17.79	7.72	4.34
Available P ($\mu g\ g^{-1}$)	0.47 - 15.41	5.08	3.52
Exchangeable K (meq $100\ g^{-1}$)	0.41 - 2.10	1.29	0.46
Exchangeable Ca (meq $100\ g^{-1}$)	3.88 - 15.92	10.15	3.03
Exchangeable Mg (meq $100\ g^{-1}$)	0.08 - 7.00	2.49	1.54
DTPA- Fe ($\mu g\ g^{-1}$)	1.00 - 36.01	9.35	9.15
DTPA- Zn ($\mu g\ g^{-1}$)	0.02 - 2.11	0.31	0.35
DTPA- Cu ($\mu g\ g^{-1}$)	0.43 - 10.04	1.57	1.77
DTPA - Mn ($\mu g\ g^{-1}$)	2.10 - 35.81	8.35	7.39

suggested BSR as a useful parameter in measuring a soil's biological activity. The relationship between these two parameters can be used for evaluation of biological activities in soils and it is expected that the relationship between biological activities and *Azotobacter* spp. population is positive. Therefore, an increase in C_{mic} generally causes in increase in *Azotobacter* spp. population, as well. This study we received that there was a statistically significant positive relationship ($p<0.05$) between *Azotobacter* spp. population of soils and C_{mic}. Whereas the relationships between *Azotobacter* spp. population of soils and BSR was found to be statistically insignificant.

The DHA reflects the total range of oxidative activity of soil microflora and, consequently it may be a good indicator of total microbiological activity (Skujins, 1973; Singh and Rai, 2004). The CA is based on the rates of oxygen release from the added hydrogen peroxide, and may be related to the metabolic activity of aerobic organisms (Glinski *et al.,* 1986; Kizilkaya *et al.,* 2004). In this study, the relationship between *Azotobacter* spp. population and CA was found to be statistically insignificant, while statistically significant important positive relationship between *Azotobacter* spp. populations of soils and DHA was observed as expected. The probable reasons for insignificant relationship between CA and *Azotobacter* spp. may be high level of moisture in soils, collection method of soil samples, and low amount of free oxygen in atmosphere covering the soil.

The GA, UA, APA and ASA are good markers of biological fertility since they are involved in microbial cycling of C, N, P and S. The GA catalyzes the hydrolysis of β-D-glucopyranoside and is one of the three or more enzymes involved in the saccharification of cellulose (Bandick and Dick, 1999; Turner *et al.,* 2002). The UA is

Table - 3: Descriptive sampling information and microbiological properties of soils

No.	Latitude	Longitude	Altitude	C_{mic}	BSR	DHA	CA	GA	UA	APA	ASA	*Azotobacter* spp. cell no. CFU g^{-1}
1	N 41° 13.309'	E 036°38.014'	15	1117.92	377.94	389.48	2.61	48.67	17.63	23.62	73.80	37
2	N 41° 28.226'	E 036° 04.591'	93	1392.09	492.46	765.68	5.16	129.95	43.78	73.69	81.32	3040
3	N 41° 35.368'	E 035° 50.254'	67	918.96	334.15	31.16	3.05	61.13	49.83	9.56	79.96	-
4	N 41° 39.286'	E 035° 27.127'	27	1285.50	331.36	445.14	5.68	110.53	53.88	16.99	76.71	-
5	N 41° 03.465'	E 035° 59.124'	660	990.32	270.13	106.88	3.98	70.10	29.31	7.41	61.60	-
6	N 40° 59.825'	E 035° 45.024'	703	592.44	303.45	146.38	3.31	68.85	13.30	19.01	73.95	-
7	N 41° 02.440'	E 035° 32.660'	587	979.82	460.93	213.51	3.51	66.29	33.66	23.37	73.34	80
8	N 41° 09.944'	E 035° 26.350'	317	1364.70	415.92	587.49	5.25	59.19	15.44	33.83	83.89	-
9	N 40° 53.265'	E 036° 04.191'	929	1631.21	351.47	832.05	4.65	78.92	30.37	23.00	69.22	-
10	N 41° 04.879'	E 037° 19.084'	10	896.11	194.26	209.90	2.37	46.36	17.60	7.95	63.66	-
11	N 41° 02.100'	E 037° 36.219'	29	1635.98	523.96	1230.37	8.10	191.49	107.54	72.87	75.11	-
12	N 40° 48.975'	E 037° 32.199'	1142	921.89	212.25	199.87	5.39	36.14	14.66	14.11	64.81	20
13	N 40° 54.864'	E 037° 30.404'	475	980.63	551.70	890.11	6.81	172.99	80.69	36.92	63.04	-
14	N 41° 04.595'	E 037° 06.145'	126	1167.24	364.97	243.55	3.18	63.84	19.16	18.25	60.15	-
15	N 40° 56.626'	E 038° 15.595'	13	723.41	350.77	525.94	3.28	61.96	19.29	16.91	62.32	887
16	N 40° 54.026'	E 038° 21.387'	27	642.34	406.15	429.66	3.27	58.99	16.73	18.47	74.07	110
17	N 41° 41.054'	E 035° 19.754'	172	865.75	306.36	359.84	4.70	84.35	18.60	21.59	62.27	197
18	N 41° 46.565'	E 034° 56.689'	746	658.16	191.96	153.66	4.36	75.06	28.51	10.19	56.70	173
19	N 41° 53.586'	E 034° 56.209'	141	1071.96	252.90	444.73	2.55	64.81	33.67	11.04	57.37	50
20	N 41° 24.688'	E 034° 59.844'	230	1334.96	275.01	237.15	3.37	37.32	32.63	8.59	60.68	130
21	N 40° 47.283'	E 035° 38.795'	473	866.60	282.33	581.86	3.77	119.37	29.32	13.89	58.83	23
22	N 40° 51.520'	E 035° 25.675'	684	1347.87	291.96	222.13	2.08	30.28	60.72	13.75	65.21	347
23	N 40° 44.916'	E 036° 21.157'	229	914.87	337.12	556.78	2.67	98.08	41.57	12.83	56.94	-
24	N 40° 33.580'	E 035° 51.989'	490	1223.13	329.88	491.14	2.53	74.90	53.20	8.70	57.45	233
25	N 40° 44.607'	E 035° 45.065'	448	622.82	508.40	99.25	7.96	88.67	17.63	8.27	61.57	-
26	N 40° 47.805'	E 035° 27.464'	517	1465.68	225.89	604.73	4.26	71.64	71.10	24.52	82.88	1677
27	N 40° 52.548'	E 035° 33.100'	582	1133.67	403.11	367.69	4.42	55.44	46.20	24.95	59.27	-
28	N 40° 51.973'	E 035° 16.913'	767	1116.92	203.97	520.73	3.75	81.86	17.24	25.70	68.86	-
29	N 40° 46.630'	E 035° 00.699'	663	1078.57	339.04	76.92	4.30	46.51	45.04	22.67	55.88	-
30	N 40° 40.154'	E 036° 37.451'	232	928.17	320.81	500.69	4.71	75.81	23.85	30.48	58.12	-
31	N 40° 29.676'	E 036° 55.888'	315	1137.38	278.51	634.48	3.47	98.73	27.36	28.42	59.94	-
32	N 40° 26.716'	E 037° 05.189'	355	702.90	327.42	198.77	1.68	26.08	62.02	24.26	62.59	313
33	N 40° 31.446'	E 036° 51.354'	768	667.30	382.27	175.36	1.71	36.94	15.62	20.83	62.85	317
34	N 40° 20.022'	E 036° 28.005'	595	965.09	244.91	340.36	2.10	35.09	27.54	11.50	63.10	117
35	N 40° 25.318'	E 036° 06.955'	532	736.43	268.59	441.39	3.79	39.18	14.51	21.98	64.37	27
36	N 40° 10.579'	E 035° 39.467'	750	704.50	161.25	437.57	2.94	47.98	21.49	18.01	58.68	297
37	N 40° 18.630'	E 035° 55.155'	731	754.83	295.14	706.03	3.19	103.74	20.32	19.92	61.50	893
38	N 40° 58.689'	E 034° 46.796'	408	394.91	188.35	49.59	1.29	21.62	29.53	10.89	60.65	17
39	N 41° 06.840'	E 034° 31.794'	383	1307.82	414.84	619.26	7.48	219.77	25.71	24.87	65.88	667
40	N 40° 50.759'	E 034° 23.524'	890	905.66	128.56	465.55	3.04	62.71	92.21	20.86	66.30	33
41	N 40° 39.684'	E 034° 31.717'	614	793.54	346.65	239.40	4.72	62.53	18.97	23.86	58.04	453
42	N 40° 35.635'	E 035° 03.455'	1168	877.11	121.34	327.50	4.23	63.28	79.82	13.75	66.37	-
43	N 40° 32.444'	E 035° 12.377'	949	912.04	369.27	218.14	4.91	48.41	53.13	15.67	63.23	-
44	N 40° 25.948'	E 034° 47.802'	777	704.51	416.51	235.72	3.99	75.06	21.79	18.95	62.11	-
45	N 40° 11.789'	E 034° 49.379'	943	1051.91	285.59	584.14	3.32	58.40	17.15	21.03	58.40	-
46	N 40° 10.760'	E 035° 08.007'	936	1028.78	318.13	318.74	2.78	57.87	14.90	15.74	64.49	40
47	N 41° 01.168'	E 034° 08.369'	532	728.79	378.47	106.59	2.02	49.89	21.36	13.05	59.39	-
48	N 41° 07.701'	E 034° 04.202'	1634	507.80	201.03	507.60	3.77	67.62	76.02	14.75	60.02	1103
49	N 40° 57.081'	E 034° 12.572'	1520	793.43	380.75	114.54	3.18	46.34	21.20	17.21	60.19	20
50	N 40° 10.537'	E 035°21.349'	699	1315.51	199.35	198.79	2.63	50.43	15.63	17.16	61.07	927

C_{mic}: Microbial biomass carbon, μg C g^{-1}; BSR: Basal soil respiration, μg CO_2-C g^{-1}; DHA : Dehydrogenase activity, μg TPF g^{-1}; CA: Catalase activity, ml O_2 g^{-1} ; GA: β-glucosidase activity, μg *p*-NP g^{-1}; UA: Urease activity, μg N g^{-1}; APA: Alkaline phosphatase activity, μg *p*-NP g^{-1}; ASA: Arylsulphatase activity, μg *p*-NP g^{-1}

Table - 4: Nitrogen fixing capacity by *Azotobacter* spp. strains for Ashby culture (without agar) and different texture group of soil

Soil no.	Isolate no.	Ashby media, µg N ml^{-1}	Soil 1 (SCL), µg N g^{-1}	Soil 2 (C), µg N g^{-1}	Soil 3 (L), µg Ng^{-1}
1	1	10.07	12.36	6.06	10.02
1	2	10.07	12.85	6.32	10.90
2	1	12.59	14.04	6.75	11.89
2	2	6.69	14.44	6.23	11.18
7	1	9.15	14.81	5.91	11.79
7	2	7.00	14.83	6.10	11.43
12	1	12.10	14.01	5.67	9.52
12	2	11.30	12.39	5.92	11.68
15	1	12.53	6.51	4.98	10.26
16	1	11.21	13.98	4.78	10.87
16	2	10.07	12.85	5.15	10.26
17	1	5.34	13.36	6.32	9.58
17	2	9.58	13.40	6.09	11.29
18	1	6.33	14.81	5.79	10.75
18	2	8.97	12.27	5.62	12.57
19	1	3.68	12.58	6.41	10.32
19	2	3.93	13.17	5.87	10.43
20	1	6.39	12.80	4.83	11.60
20	2	6.63	12.81	5.39	10.91
21	1	3.62	11.88	5.48	11.60
21	2	5.16	13.35	5.73	11.88
22	1	5.10	12.67	5.86	12.10
22	2	3.50	13.71	5.40	13.19
24	1	7.31	12.18	5.41	10.78
24	2	6.26	12.22	5.65	9.93
26	1	5.47	12.81	4.97	12.23
26	2	5.76	13.72	5.63	11.07
32	1	7.61	12.90	5.41	10.22
32	2	9.15	14.10	5.00	10.32
33	1	6.63	13.95	6.37	11.79
33	2	7.80	14.00	7.09	11.91
34	1	5.96	14.31	6.00	11.97
34	2	13.69	14.00	6.19	11.09
35	1	8.47	13.49	6.11	10.49
35	2	11.30	12.09	5.94	9.03
36	1	7.55	13.03	5.40	10.36
36	2	8.17	13.13	5.83	10.68
37	1	14.98	11.73	5.60	11.52
37	2	9.33	14.18	5.70	11.11
38	1	8.23	12.84	4.86	12.77
38	2	11.32	13.90	6.40	11.65
39	1	10.81	14.40	5.76	9.54
39	2	11.58	14.77	9.21	10.46
40	1	17.55	16.42	12.94	13.27
40	2	15.09	15.58	12.47	11.30
41	1	29.35	15.38	12.29	12.42
41	2	23.23	15.37	13.81	12.13
46	1	14.00	15.49	11.36	12.26
46	2	21.37	16.33	13.52	13.15
48	1	15.04	15.23	14.13	13.47
48	2	13.63	12.50	14.29	11.74
49	1	13.47	14.98	14.38	12.89
49	2	12.40	15.65	13.36	12.04
50	1	14.80	16.60	15.91	11.87
50	2	14.98	15.99	14.59	12.07

SCL : Sandy clay loam, C : Clay, L : Loam

Table - 5: Physico-chemical properties of isolated *Azotobacter* spp. strains that have the most N fixation

Soil physico-chemical properties	Soil number					
	40	41	46	48	49	50
Vegetation type	Wheat	Wheat	Wheat	Forest	Pasture	Wheat
Soil temperature (°C)	26.00	24.50	23.40	13.10	22.60	23.40
Air temperature (°C)	31.00	34.10	33.50	28.10	28.80	30.60
Soil moisture (%)	19.41	16.97	21.48	13.91	14.65	13.90
Air moisture (%)	49.50	28.40	43.00	38.10	37.30	52.30
Sand (%)	70.95	21.19	27.76	51.18	56.71	28.92
Silt (%)	16.94	23.88	24.08	34.88	27.34	32.10
Clay (%)	12.11	54.93	48.16	13.94	15.95	38.98
Texture class	SL	C	C	L	SL	CL
pH (1:1)	7.78	7.68	8.00	4.90	7.78	7.90
EC(dSm^{-1})	0.59	0.47	0.23	0.15	0.41	0.39
$CaCO_3$ (%)	2.40	18.63	14.58	< 0.01	0.75	9.44
Organic matter (%)	2.70	3.41	7.68	11.88	3.94	2.76
Total N (%)	0.22	0.17	0.29	0.63	0.26	0.22
C:N ratio	7.12	11.63	15.36	10.94	8.79	7.28
Available P ($\mu g\ g^{-1}$)	1.32	3.02	1.78	7.26	2.99	1.30
Exchangeable K (meq 100 g^{-1})	1.08	1.82	1.68	0.86	0.48	1.80
Exchangeable Ca (meq 100 g^{-1})	13.63	13.54	12.96	3.88	10.67	10.21
Exchangeable Mg (meq 100 g^{-1})	6.29	2.54	3.04	0.67	1.63	1.75
DTPA- Fe ($\mu g\ g^{-1}$)	3.18	1.04	1.41	36.01	4.33	2.23
DTPA- Zn ($\mu g\ g^{-1}$)	0.66	0.74	0.50	2.16	0.88	1.01
DTPA- Cu ($\mu g\ g^{-1}$)	0.33	0.17	0.15	0.19	0.16	0.16
DTPA – Mn ($\mu g\ g^{-1}$)	5.36	4.76	4.87	35.81	8.91	5.54

SL : Sandy loam, C : Clay, L : Loam, CL : Clay loam

involved in the hydrolysis of urea to carbon dioxide and ammonia, which can be assimilated by microbes and plants. It acts on carbon-nitrogen (C-N) bonds other than the peptide linkage (Bremner and Mulvaney, 1978). The APA hydrolyzes compounds of organic phosphorus and transforms them into different forms of inorganic phosphorus, which are assimilable by plants (Amador *et al.,* 1997). Arylsulphatase (ASA) is the enzyme involved in the hydrolysis of arylsulphate esters by fission of the oxygen-sulphur (O-S) bond. This enzyme is believed to be involved in the mineralization of ester sulphate in soils (Tabatabai, 1994). This study suggested positive relationships between extracellular enzymes (GA, APA and ASA), with the exception of UA, and *Azotobacter* spp. population were found ($p<0.05$). While taking nourishment process into account, *Azotobacter* spp., which is considered to be heterotrophic, supplies C that it needs from organic C components located completely in soil environment. Also, it supplies N from free N in atmosphere and nourishment substances again from soil, which is considered to be its life source. However, in the presence of inorganic N in environment, *Azotobacter* spp. can use this N as well and as a result, their, especially N fixation's, effectiveness diminishes, which means that N is an organism supplying its nourishment substances from soil in normal conditions. Since the determination of positive relationship between *Azotobacter* spp. population and extracellular enzymes providing breakdown and absorption of both nourishment substances in soil and organic components of these nourishment substances, the results of this study is expected. The reason for the finding of insignificant relationship between UA and *Azotobacter* spp. population couldn't is a consequence of usage of inorganic components containing N compounds exposed as a result of UA effect by *Azotobacter* spp., their activity and its loss of agents, such as N fixation, in significant level. It is generally assumed that little N-fixation will occur when sufficient amounts of either ammonium or nitrates are present in the soil. Urea has been reported to retard fixation markedly, and N_2O is a specific competitive inhibitor (Stewart, 1966). However, setting critical levels only on the basis of the actual amounts of N in the soil is not possible. Clearly more complex C:N:P:S ratios are involved in regulating the nitrogen fixation. Bremner and Shaw (1958), and Greenland (1962) found that when the C:N ratio in the soil is less than 5, no fixation of nitrogen might be expected. This study revealed that C:N ratio of soil samples study was 7.72 on average and ranged from 1.73 to 17.79. Where C:N ratio of 15 soil samples was under 5, resulting in insignificance relationship between UA and *Azotobacter* spp. population.

Nitrogen fixing capacity by native *Azotobacter* spp. strains in pure culture and soil: To determine N fixation capacities of local *Azotobacter* spp. strains in pure culture, isolated from soils, their N fixation capacities were measured in liquid Ashby Media by isolating more than 1 colony (total 55 isolates) from *A. chroococcum* strains present in 28 soil samples. It was explored that N fixation capacities of *Azotobacter* spp. strains grown in pure culture are resemble. However, *Azotobacter* spp. strains grown after isolating from soil samples of 40, 41, 46, 48, 49 and 50 had the highest ability of N fixation in pure culture environment compared to others. On the other hand, N fixation capacities of *Azotobacter* spp. strains in soil

environment were examined by inoculation of *Azotobacter* spp. strains isolated from the soil samples to soils with different textural properties in laboratory conditions and their incubation for 8 weeks in optimal environmental conditions. Based on the research results, there were considerable variances in N fixation capacities between isolated *Azotobacter* spp. strains and the soil where it is located. It was also found that *Azotobacter* spp. strains had more N in sandy clay loam soils than that of loam and clay soils. The soil pore system which consists of various amounts of water and air has to be characterized quantitatively in order to describe the soil as a habitat for *Azotobacter* spp. population and their activity. Soil pore size as determined by soil texture may be as important as the size distribution for the transport of gases and nutrients (Stotzky, 1972). This study also addressed the physical properties of differently textured soils in undisturbed and remolded state and their effect on N fixation by different *Azotobacter* spp. strains. Research results showed that the most N fixation by *Azotobacter* spp. was in coarse-textured (sandy clay loam) soils. The probable reason is the water and air rapidly permeates coarse soils with granular subsoil, which tend to be loose when moist and don't restrict water or air movement (Haris, 1981).

Considerable variations in all *Azotobacter* spp. strains were found for the different inoculated isolates in fine-textured soil (clay soil). The basic reasons for this are increasing surface area and nutrient contents (Stotzky, 1972; Haris, 1981). Clay, the smallest sized particle (<0.002 mm), has the greatest surface area. Clay particles have a million fold more surface area per mass than silt. Clay is capable of holding large amounts of water and nutrients such as P, K (Table 1) but may prevent the release of water for *Azotobacter* spp. use. Besides, *Azotobacter* spp. strains realized the most N fixation, which is a similar process in pure culture conditions, in the soil samples of 40, 41, 46, 48, 49 and 50. The physico-chemical properties and vegetation types of these soil samples are given in Table 3 and 5 in detail.

This study was conducted in order to expose relationships between soils' microbiological properties and *Azotobacter* spp. population isolated from Northern Anatolia soils and to explore *Azotobacter* spp. strains' N fixation both in pure culture environment and in soils with different textural properties. It was fixed that there were differences among native *Azotobacter* spp. strains adapted to the conditions of local environments in terms of their N fixation capacities. Based on the results of the research, it is inevitable that these strains, which have high in N fixation capacity in soils, should be used as fertilizers that will decrease environmental pollution sourced chemical fertilizers containing N in agriculture. Hence, they may be significantly contributed to save due to substitution for chemical fertilizers would. However, further research should be conducted for determination of general characteristics of *Azotobacter* spp. strains and DNA-RNA line-ups of strains with high N fixation capacities, determination of their effect on plant growing by implementation of these strains to soils in arable field and greenhouse conditions, and study of their potential ecology-friendly implementation are necessary.

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