SCREENING AZOTOBACTER SPP., BIOAVAILABILITY FROM FOUR ECOLOGICAL SYSTEMS IN ZAKHO, KURDISTAN REGION – IRAQ

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ABSTRACT:

This project was designed to analyse the impact of several environmental factors on the *Azotobacter* abundance in soil samples. *Azotobacter* spp. were isolated from four ecological systems and screened for calculating CFU numbers per two grams of soil. Soil samples with a range of textures were collected from Zakho town (Kurdistan Region – Iraq). Soil particle sizes distributions were measured as (% sand, % silt and % clay). Soil chemical and physical features like (pH, electrical conductivity, organic matters availability) in addition to various soil textures; Loam (4 samples), clay (3 samples), silt (1 sample), clay loam (4 samples) were determined. Electrical conductivity measurements were fallen between 35.7 dSm^{-1} in sample (L2) and 5.17 dSm^{-1} in sample (C2). Organic matters availability was from 17.6% in sample (C2) to 4.18% in sample (R3). Soil samples' pH values were between 9.41 as in sample (R2) and 7.72 that found in sample (L3). Concerning all growth impact factors, the maximum number (370 CFU) of *Azotobacter* was observed in the R1 sample. No *Azotobacter* growth was observed in samples; G1 and G2 while sample G3 produced (55 CFU). All isolates show stable growth from the legume rhizosphere zoon (L1 105-, L2 60-, and L3 125-CFU). No isolates were obtained from the R2 soil sample while only 70 CFU were seen in the R3 sample. Samples; C1 and C3 formed 94 and 108 CFU respectively whereas no isolated colonies were noticed in the C2 soil sample.

KEYWORDS: Soil, soil texture, soil bacteria, *Azotobacter*, legume, pH, bacterial density. Zakho, Kurdistan-Region, Iraq.

1. INTRODUCTION

Bacterial species belong to *Azotobacter* genus are Gram-negative and usually motile, spherical or oval in shape forming thick-walled cysts (Dar *et al*., 2021). *Azotobacter* spp are heterotrophic bacteria, aerobic and playing a crucial role in atmospheric nitrogen fixation (Geisler *et al*., 2020). *Azotobacter* was usually found in alkaline soil, water, and in a symbiotic association with some plants (Kumar *et al*., 2007).

Several factors – in different levels – regulate the abundance of *Azotobacter* spp such as the bioavailability of organic matter, soil depth, soil moisture in addition to physiochemical reactions and bacterial interface properties (Aasfar *et al*., 2021).

Azotobacter genus contains a number of species like; *A. nigricans*, *A. tropicalis*, *A. paspali*, *A. agilis*, *A. beijerinckii*, *A. macrocytogenes*, *A. armeniacus*, *A. vinelandii*, *A. insignis*, and *A. choococcum* which are considered to be the genus representatives and most species have been isolated and studies (Kumar *et al*., 2007).

Beside the soil electrical conductivity, soil acidity plays a major role in the population of *Azotobacter* at any ecological system where the growth of *Azotobacter* is dramatically declined in acidic pH. In some cases, the growth of *Azotobacter* may completely inhibit in pH under 6 (Andjelkovic *et al*., 2018). It was established that the best pH value for the *Azotobacter* development was determined to be between 7-7.5 (Becking, 2006). Some species of *Azotobacter* like *A. chroococcum* can grow at pH 9 and even above 9 while *A. salinestris* show poor growth at this pH value (Aasfar *et al*., 2021). *Azotobacter* spp boost crop growth and production via some vital processes like; biosynthesis of biologically energetic elements, motivation of rhizospheric bacteria, and production of plant-disease related microbes' inhibitors, inducing; nutrient uptake and increasing the bio-availability of soil nitrogen (Lenart, 2012). Studies on *A. chroococcum* in crops production have confirmed the significance of this bacterium in enhancing plant nutrition uptake and improving of soil fertility (Kurrey *et al*., 2018).

Numerous studies focused on the relationship between the *Azotobacter* species population and the presence of the nutrient elements in soil like; potassium, phosphorous and calcium along with the attendance of the soil-organic matters (Kizilkaya, 2009; Imtiaz-Rashid, *et al*., 2016; Ali, *et al*., 2007). The correlation between *Azotobacter* population, biochemical activities, microbial mass, basal soil respiration, enzymes activities like: urease, glucosidase, dehydrogenase, phosphatase, catalase, and sulphatase on a side and the ecosystem features like; soil quality, soil health and fertility on the other side have also been analyzed (Kizilkaya, 2009). Nevertheless, the adaptation of the *Azotobacter* spp. to a range of environmental factors that positively and negatively determine the bacterial growth rate has not been previously investigated in Kurdistan region-Iraq. Hence, the current work tried to focus on the assessment and compression of the *Azotobacter* population in several ecological systems with different soil texture natures along with dissimilar soil features and contains.

2. MATERIALS AND METHODS

All the experiments mentioned in this work were repeated for two or three times according to the nature of the test and the average measurements were considered.

2.1 Collection and Preservation of the Soil Samples

In the current work, the soil-habitants *Azotobacter* spp. were isolated and analyzed from several soil samples in the environmental microbiology laboratory - Environmental Sciences Dept., Faculty of Sciences, University of Zakho, Kurdistan region – Iraq. Soil samples were collected from four ecosystems: legumes crop field, grass land, citrus rhizosphere zoon, and plant-free red-soil land. During the soil samples collection, different tools were used such as; plastic bags, spatula, alcohol, and knives. Around 2 kg were collected from each determined point, stored in polyethylene bag and marked. The soil samples were collected either from top 4 cm of the soil profile or around the plant rhizosphere as previously mentioned (Islam *et al*., 2008).

2.2 Classification of Soil Samples

For each of the four soil samples, samples were collected from three locations (12 samples in total) and screened individually. Therefore, samples were classified according to the type of the soil origins. "Grass-planted land" group samples were named as; G1, G2, and G3. Samples from the "Legume-rhizosphere zoon" were categorized as; L1, L2 and L3. Collected samples from the "plant–free Red-soil" were characterized as: R1, R2, and R3. Finally, **"Citrus-planted land"** (*Citrus aurantium*) samples were named; C1, C2, and C3.

2.3 Sample Storage and Processing

Two grams from each soil sample were incubated with 50 mL of Luria-Bertani (LB) broth medium, shacked for 10 min with aeration. 100 µL were then transferred onto *Azotobacter* specific (mannitol) agar medium; sodium chloride 0.2 g/L, dehydrated sodium citrate 2.0 g/L, heptahydrated magnesium sulfate 0.2 g/L, ammonium dihydrogen phosphate 1.0 g/L, dipotassium hydrogen phosphate 1.0 g/L, ferrous sulphate (trace), bromthymol blue 0.08 g/L, mannitol 20.0 g/L, agar-agar 15.0 g/L, and dH2O up to 1L. Medium pH was adjusted to 8.3 ± 0.2. This medium is recommended for the isolation and cultivation of *Azotobacter* spp. from soil (Ambesh *et al*., 2017). For each sample, colony-forming units (CFU) were calculated and then stored at 4ºC for biochemical investigation along the experiment processes.

2.4 Preparation of Microbiological Media

All bacterial media components were prepared as described by Sambrook *et al*. (2001) or according to the manufacturer instructions. For each medium, the components were weighted by an electronic balance. Around 750 ml of dH2O was added and mixed with heating on a magnetic stirrer hot plate. pH was adjusted by adding either HCl or NaOH when required, the final volume was adjusted to 1L. Medium was then sterilized by autoclaving at 121˚C, 20 lb/in^2 for 20 min. Finally, the medium was poured into sterile Petri-plates. Post inoculation and all plates were aerobically incubated at 28-30 ºC for 3-5 days.

2.5 Determination of Field Soil Texture

40 gm of oven dried soil (sieved with 2mm sieve) was put in a 500 ml beaker, then 10 ml of H2O2 (6%) was added to the soil. The suspension was left on the bench until frothing ceases and another 10 ml of H2O2 was added again. The suspension was transferred to a dispersing cup until there was no further reaction with fresh H2O2. Finally, another 10 mL of sodium hexametaphosphate 5% was added and mixed with 400 mL of water. The suspension volume was adjusted to IL, then quantitatively transferred into a sedimentation cylinder and further mixed for 5-10 min by a plunger. The hydrometer was inserted into the suspension and the first reading was recorded as (R1) after 40 sec. The hydrometer was removed and the first temperature was recorded as (T1). The above readings

determine the quantity of the silt and clay in the suspension. After two hours, the above readings were calculated again as R2 and T2 in the same manner. The suspension was prevented from any shaking. These couple of readings assesses the weight of the clay (in grams) per liter still in the suspension (Mwendwa, 2022).

2.6 Determination of the Soil Acidity (pH)

The *METTLER TOLIDO* "FE20 Five Easy" pH measurement tool (*Fisher Scientific*) was used as previously described by Mikolajewicz and Komarova (2019). The soil solution was prepared by adding 1 gm of soil into 2.5 mL of dH2O in a clean 250 mL flask. The electrode was sunk in the solution and left for 2 min, then followed by the recording of the pH value.

2.7 Measurement of the Soil Electrical Conductivity

Based on the Rysan and Sarec (2008) methodology. One gm of the soil sample was added to 2.5 mL of dH2O and the conductivity was determined by using the conductivity tool *METTLER TOLIDO* "FE20 FiveEasy" (*Fisher Scientific*). The solution was mixed well and the electrode was put in the solution and left for 2 min. This was followed by recording the level of the electrical conductivity in milliSiemens per meter (mS/m^{-1}).

2.8 Determination of the Soil-Organic Matter

Soil-organic matter was performed as illustrated by (Rice *et al*., 2021). An empty, clean, and dry porcelain dish was weighted (P) to determine the mass of the dish (W$_A$). This was done by adding 10 gm of the soil into the porcelain dish to obtain the weight of the soil sample (W$_{DS}$). The soil sample containing dish was dried in the oven at 550 °C for an hour so as to red-off the soil moisture. The porcelain then was taken out from the oven and left on the bench at room temperature to cool down. Finally, the soil-containing ash (W$_o$) was determined by grams. The organic matter of each soil sample in this work was calculated according to the following equation:

OM = {Wo / W$_{DS}$} * 100
Where Wo = W$_{DS}$ - W$_A$, and W$_A$ = W$_{PA}$ - W$_P$

2.9 Identification of the Isolates

2.9.1 Gram Staining: Soil-isolated bacteria were stained with Gram-stain as described by (Colco, 2005).

2.9.2 Bacterial Motility Test: LB sterile semi-solid medium – containing 0.75% (w/v) agar-agar – in a test tube was used for the investigation of the isolates motility as mentioned before (Eric and Thomas, 2009). A sterile needle was well-touched with fresh grown *Azotobacter* colonies (18-24 h) on the *Azotobacter*-specific agar medium, the semi-solid medium was then stabbed by *Azotobacter*-contaminated needle into a depth of 4-6 cm. Inoculated test tube was then incubated with aeration at 30 °C for 48-96 h.

2.10 Biochemical Analysis of the Isolates

2.10.1 Bacterial Glucose Fermentation: Establishing the production of the acetylmethyl carbinol from glucose was carried out via Voges-Proskauer test. A loop-touch of *Azotobacter* was taken from a fresh colony and lightly inoculated into the Methyl Red-Voges-Proskauer (MR-VP) broth medium, followed by aerobic incubation at 30 ºC for 4-5 days. Post incubation period, 2 mL of the broth were aliquot to a clean test tube and re-incubated for another 24 h. Six drops of 5% alpha-naphtol were added into the medium and mixed well to aerate.Another two drops of 40% KOH were added and mixed well again. The tube was then shacked vigorously for 30-min, a pink-red color at the surface of the tube was considered as a positive result.

2.10.2 Hydrogen Peroxide Naturalization: Decomposition of H2O2 to H2O and O2 via the catalase enzyme (Lima *et al*., 2020)

was determined by putting microscope slide inside a Petri-dish to limit catalase aerosols. A loop touch of *Azotobacter* spp. from a fresh (48-72h) isolated colony was transferred by a sterile loop and placed onto the microscope slide with avoiding agar transferring onto the slide. A drop of 3% H_2O_2 was added to the inoculum on the slide by using a dropper (MacFaddin, 2000). Forming of bubbles was checked up against a dark background to enhance readability.

2.10.3 Citrate Utilization Test: The ability of *Azotobacter* species to use the citrate as the sole source of carbon and ammonium dihydrogen phosphate as the sole source of nitrogen (Noar *et al.*, 2015) was confirmed by preparing a slant Simmon's citrate agar in a test tube which was streaked with a light inoculum picked from the center of a fresh isolated colony. The test tube was immediately incubated aerobically at 30 °C± 2 °C for 4 days. Color changing from green to blue along the slant was checked up and accounted as a positive result.

2.10.4 Tryptophan Decomposition: The capability of *Azotobacter* spp. to decompose tryptophan was performed as described by Sumbul *et al.* (2020). A loop-touch from a fresh colony was inoculated in a test tube containing fresh 4 mL of tryptophan broth then aerobically incubated at 30 ºC for 24-48 h followed by adding 0.5 mL of Kovac's reagent into the broth culture. The presence or absence ring was checked up.

2.10.5 Urea Hydrolysis: Heavy inoculation from a fresh *Azotobacter* colony was inoculated on the entire slant surface of the urea agar in a test tube with avoiding the butt stubbing to keep it as a color control (Vitolo, 2022). Color change observation was carried out for up to 5 days. Urease production is indicated by a bright pink color on the slant.

3. RESULTS AND DISCUSSION

3.1 Estimation of the Soil Samples Acidity

Soil pH is an indicator of the concentration of hydrogen ions in the soil samples (Thomas, 2018). Soil acidity is increased due to secreting of H_2CO_3 as a final product composed from CO_2 that is produced by the decomposition of the organic matters in the soil by the soil-mediated microorganisms. Soil acidity is also elevated as a result of hydrogen releasing through plant metabolism of some important chemical compounds such as; NO^{-3}, NH^{+4}, Ca^{2+}, and H_2PO^{-4} (Neina, 2019). On the other side, the accumulation of several soil-derived element cations effectively contributes to the soil alkalinity as in the case of sodium, potassium, magnesium, and calcium. The above cations are released from macro chemical compounds like; aluminosilicates and silicates, throughout natural chemical reactions (Bloom and Skyllberg, 2012). The concentration of the hydrogen ions usually increases when the total alkalinity increases. However, the balance of soil pH relatively remains in a specific range, as the accumulation of cations like calcium or sodium increases the soil alkalinity and encourages other elements to raise the soil acidity. For example, increasing of a determined amount of sodium in an alkaline soil induces the dissolution of calcium carbonate, which – in turn – declines the pH value (Bloom and Skyllberg, 2012). *Azotobacter* species prefer slightly alkaline ecosystems with a pH range 7.0 – 7.5 as this spectrum of alkalinity supports the physiological functions and abundance of *Azotobacter* spp. that could fall between 10^2 and 10^4 colony form units per gram of soil (Becking, 2006). All the soil samples used in this work were found to be alkaline with a pH array from 7.72 as in sample (L3) to 9.41 as in sample (R2). As groups, the highest alkaline soil samples were found in the C-group, G-group, L-group, and then R-group respectively. The above pH values were also reported by previous Iraqi-soil pH measurements (Umer, et al., 2012). Maximum bacterial Colony Form Units (CFU) growth was obtained from the R1 sample (CFU 370, pH 8.43), while lower bacterial growths were found in above or close to pH 9 as in

cases; G3 (CFU 55, pH 9.12), R3 (CFU 70, pH 8.69), C1 (CFU 94, pH 8.96) and C3 (CFU 108, 9.27) (Table 1). Based on the above findings, it appears that *Azotobacter* prefers alkaline conditions for better growth, however, extreme soil alkalinity clearly decreased the bacterial growth rates and declined the bacterial multiplication mediated metabolism which was reflected on the number of produced CFU. The findings were in harmony with a recent study which confirmed the *Azotobacter* spp. sensitivity to pH 9 or above where only few species of *Azotobacter* were capable of growing in pH 9 or higher (Aasfar *et al.*, 2021). The above concept was even more obvious by the absence of any *Azotobacter* growth close or above pH 9 as in cases; G1 (pH 8.99), G2 (pH 9.16), R2 (pH 9.41) and C2 (pH 9.26) which was in an agreement with previous work (Aasfar *et al.*, 2021) (Table 1). Sample R1 with the highest bacterial growth rate was also isolated from the clay-loam soil texture which was the best soil type for *Azotobacter* growth (Kizilkaya, 2009). In this experiment, two factors; relatively optimum pH conditions and the nature of soil texture were contributed and determined the *Azotobacter* growth rate.

3.2 Impact of Soil Electrical Conductivity

Electrical conductivity (EC) and pH value are the most correlated factors to the bacterial abundance in soil (O'Brien *et al.*, 2019). The highest EC was found in sample L2 (35.7 dSm^{-1}) which was around seven-folds higher than the lowest EC observed in sample C2 (5.17 dSm^{-1}). No *Azotobacter* growth was found in the samples with EC lower than 10 dSm^{-1} as in the cases G1, G2, R2 and C2 with the exception of L1 (EC 8.52, 105 CFU), C1 (EC 5.24, 94 CFU), and C3 (EC 6.27, 108 CFU). Furthermore, EC values above 30 dSm^{-1} produced 60 and 125 CFUs as in the cases of L2 (EC 35.7 dSm^{-1}) and L3 (EC 33.4 dSm^{-1}) respectively. However, the highest bacterial growth was obtained from sample R1 (370 CFU) with the EC value of 22.7 dSm^{-1}. According to the above results, it seems that EC *per se* cannot be the only determining factor for the *Azotobacter* abundance in a natural ecosystem as other factors also interact and regulate the bacterial bioavailability. Keeping in mind that high EC value reflects the presence of salts in soil while the low values of EC refer to the scarcity of the soil-containing salts where both extreme values negatively affect the growth of bacteria (Othaman *et al.*, 2020). Thus, it gives impression that a highest rate of *Azotobacter* growth could be observed in a comparatively 'medium' EC value as in the case of R1 (Table 1). Nevertheless, other three factors were included in this study those interact with the EC effect on the bacterial abundance.

3.3 Impact of the Organic Matter on the Azotobacter Growth

Over years, there was an argument if the composed organic manure enriched with free-living, N_2-fixing organisms will contribute to increase the crop production more than the composed organic of similar composition but not enriched with microbes. This argument was focusing on the prominence of the nitrogen-fixing bacterial presence and the organic matter in improving the plant growth (Kumar *et al.* 2015; Rodriguesa *et al.*, 2018). Increasing the plant growth rates were reported due to mixing manures with *Azotobacter* over the same manures without nitrogen fixing-bacteria. It was, also, confirmed that the availability of *Azotobacter* in the fertilizer increased the abundance of nitrogen-containing compounds (Rodriguesa *et al.*, 2014). Thus, commercial fertilizers enriched with *Azotobacter* species or the other nitrogen-fixation bacteria have been produced in the European markets (Rodriguesa *et al.*, 2014).

The highest organic matter percentage was found in the sample G3 (17.8%) producing 4-fold higher than the lowest rate that was found in sample R3 (4.18). However, only 55 CFUs were produced in this sample while 70 CFU were observed in R3 soil

sample. No bacterial growth was noticed in the samples: G1, G2, R2, and C2 in spite of containing of 4.96, 6.14, 7.66 and 17.6% of organic matter respectively. The highest number of CFU was obtained from sample R1 (370 CFU) which contained only 7.63% of the organic matter. Samples: L1, L2, L3, C1, and C3 produced 105, 60, 125, 94, 108 CFU which contained 8.09%, 10.48%, 9.32%, 8.9%, and 5.84% of the organic matter respectively (Table 1). Bacterial abundance usually was found to be in low level in soil with scarcity of nutrient. However, in this study, the best *Azotobacter* growth (sample R1) was not in the soil that possesses the highest density of nutrients. It appears that high level of nutrients in a particular soil may support the growth of other competing bacteria which could inhibit the growth of *Azotobacter* species. Thus, the highest growth of *Azotobacter* species was not observed in the soil sample (L2) which had the maximum rate (10.48%) of the organic matters. Based on the current results, it seems that there is no any relationship between the rate of the organic matter and the abundance of *Azotobacter* (Table 1). Similar observations were reported toward the relationship between the bioavailability of *Azotobacter* and the accumulation of some specific types of organic matters such as berseem hay, farm yard manure, and wheat straw (Iqbal *et al*., 2018).

Table 1: Soil samples' chemical, physical, and biological properties.
Each sample illustrated different ecological features and different rates of bacterial growth.

Plant-Depend. Soil Sample	Grass			Legumes			Red soil (Plant-Free Soil)			Citrus		
Geographical Site/ Sample	G1	G2	G3	L1	L2	L3	R1	R2	R3	C1	C2	C3
Soil Texture Classes	Loam	Loam	Clay	Clay	Silt	Loam	Clay Loam	Clay	Clay Loam	Clay Loam	Loam	Clay Loam
Soil Samples Acidity (pH)	8.99	9.16	9.12	8.21	7.88	7.72	8.43	9.41	8.69	8.96	9.26	9.27
Electrical Conduct (dSm^{-1})	9.22	5.54	23.2	8.52	35.7	33.4	22.7	8.12	13.8	5.24	5.17	6.27
Org. Matters Abundance (%)	4.96	6.14	17.8	8.09	10.48	9.32	7.63	7.66	4.18	8.9	17.6	5.84
Number of CFU/50μL	NON	NON	55	105	60	125	370	NON	70	94	NON	108

3.4 Investigation of the Isolates

3.4.1 Morphology of Isolates: Under 100X microscope lens, isolates cells found to be 1-10μm long, rod shaped, formed thick-walled cysts and large quantities of capsular slime with cream light chain reflecting the features of *Azotobacter* species (Table 2, Supplement 1).

3.4.2 Citrate Utilization Test: Isolate use the sodium citrate as an only source of carbon and inorganic ammonium hydrogen phosphate as a sole source of nitrogen were confirmed by changing the medium color from green to blue. Samples; G2, G3, R3, and C2 did not show the above biological aspects. However, all the rest isolates were found to have the above biochemical reaction as illustrated in (Table 2, Supplement 2).

3.4.3 Glucose Fermentation: All isolates demonstrated the capability of glucose utilization by producing stable acid and changing the medium color (methyl red) from yellow to red (Table 2, Supplement 3). This biochemical reaction emphasizes the identity of the *Azotobacter* species.

3.4.4 Naturalization of Hydrogen Peroxide: All isolate bacteria presented an ability to naturalize hydrogen peroxide by decomposing it to water molecules and the appearance of oxygen bubbles through the production of the catalase enzyme suggesting – with the other biochemical reactions – that the isolates belong to *Azotobacter* genus (Table 2, Supplement 4).

3.4.5 Indole Production: All isolates were screened for the ability of the amino acid tryptophan to produce the indole ring as a by-product indicated by the appearance of a pink-reddish ring on the top of the medium. All the isolated species demonstrated the capacity of producing indole ring which supported the *Azotobacter* identity (Table 2, Supplement 5).

3.4.6 Urea Hydrolysis: For further approval of isolates identity, the isolates were subjected to urea hydrolysis test. All the isolates – with an exception of sample L1 – found to produce the urease enzyme by turning the medium's colour from yellow to pink referring to the bacterial ability to hydrolyse urea to ammonia and carbon dioxide (Table 2, Supplement 6).

3.4.7 Isolates Motility: Isolates – excluding sample R1 – demonstrated a capability of movement when inoculated in semi-solid LB agar containing 0.7 g/L agar-agar producing another aspect of the *Azotobacter* characteristics (Table 2, Supplement 7).

Table 2: Morphological and biochemical characteristics of *Azotobacte* spp. Biochemical experiments confirmed that the isolates organisms of the same species regarding that the *Azotobacter* is a Gram-negative, citrate utilizer, motile, glucose fermenter and producer of urease, indole and catalase.

Plant-Depend. Soil Sample	Grass			Legumes			Red soil (Plant-Free Soil)			Citrus		
Geographical Site/ Sample	G1	G2	G3	L1	L2	L3	R1	R2	R3	C1	C2	C3
Bacterial Appearance	1-10 μm long, rod shaped. Formed thick-walled cysts and large quantities of capsular slime with cream light chains.											
Fermentation of Glucose	+ Y→R	+ Y→R	+ Y→R	+ Y→R	+ Y→R	+ Y→R	+ Y→R	+ Y→R	+ Y→R	+ Y→R	+ Y→R	+ Y→R
Catalase (H$_2$O$_2$ Naturalization)	+ Bub	+ Bub	+ Bub	+ Bub	+ Bub	+ Bub	+ Bub	+ Bub	+ Bub	+ Bub	+ Bub	+ Bub
Citrate utilization	+ G→B	- G→B	- G→B	+ G→B	+ G→B	+ G→B	+ G→B	+ G→B	- G→B	+ G→B	- G→B	+ G→B
Indole production	+ RPR	+ RPR	+ RPR	+ RPR	+ RPR	+ RPR	+ RPR	+ RPR	+ RPR	+ RPR	+ RPR	+ RPR
Urease production	+ Y→P	+ Y→P	+ Y→P	- Y→P	+ Y→P	+ Y→P	+ Y→P	+ Y→P	+ Y→P	+ Y→P	+ Y→P	+ Y→P
Motility	+	+	+	+	+	+	-	+	+	+	+	+

Key: + (positive reaction), - (negative reaction).
Media colors change: **Y→R** (Yellow to Red), **G→B** (Green to Blue), **RPR** (Red-Pinkish Ring appearance), **Y→P** (Yellow to Pink),
Bub: appearance of bubbles.

3.5 General Discussion

Azotobacter spp. are one of the main nitrogen fixing bacteria and considerably contribute in improving the growth and enhancement of the crops on the farmlands. *Azotobacter* spp. are soil-habitant microorganisms and can be isolated from soil with diverse chemical and physical properties as seen in this study. The extensive bacterial growth on the plates impeached that the only bacteria that accomplished the fixing atmospheric nitrogen could be propagated on/in soil as in the case of *Azotobacter*. The complete range of enzymes includes nitrogenase, fendoxin, in addition to hydrogenase. Those are needed for nitrogen fixation and are available in *Azotobacter* (Amutha *et al.*, 2014). Being a main group of the soil-microbial community, *Azotobacter* plays a crucial role in soil enrichment by supplying the soil with different kinds of important biochemical substances such as vitamins, plant hormones, antifungal substances, hydrogen cyanide and siderophores. Throughout the above secondary metabolites, a major augmentation can be observed on the shoots, roots and seeds germination of a wide-range of crops. Among the soil-derived genera, *Azotobacter* spp. have the ability to decompose many types of pesticide complexes like; phenols, substituted phenolics and hazardous compounds (Elsyaed, 2013). The characteristics of colonies and the biochemical features of this work isolates illustrated that these soil isolated bacteria are Gram-negative and produced positive chemical reactions which were in harmony with some previous publications (Ujah *et al.*, 2021; Vahist *et al*, 2013). The biochemical reactions in a variety of tests differentiate the members of the family Enterobacteriacea. For instance, indole test refers to the ability of the bacteria to decompose tryptophan and produce indole. The catalase test establish the capability of the microorganism to naturalize the hydrogen peroxide into water and oxygen. Furthermore, methyl red test is applied to investigate the bacterial role to produce stable acids during the fermentation process when the bacterial medium is enriched with glucose. Soil acidity is an important environment for many biochemical activities occurring within the soil. Soil pH helps in configuring decisions regarding the kind of plants suitable for the planting site, the possibility of altering the soil pH (toward acidity or alkalinity) and a strong indicator of obtainability of nutrients in the soil (Jones *et al.*, 2013). Bacterial community is directly affected by the soil acidity. It was suggested that the nitrogen fixation is dramatically reduced in condition of pH 4.7 or below (Jones *et al*, 2013).

The pH of this study soil samples was ranged between 9.41 (R2) and 7.72 (L3) which indicated that the soil samples were strongly alkaline and may be saturated with high amount of sodium. This range of soil acidity was confirmed as a great factor inducing bacteria to fix the atmospheric nitrogen. The amount of the salts present in the soil is measured by the soil electrical conductivity. The quantity of the soil-salts is a strong sign of soil health status. This amount of salts directly affect the crops' yield, susceptibility, nutrient bioavailability and the bacterial activity which affect the major soil processes such as the emission of greenhouse gases like; NO_3, CH_3 and CO_2 (Adviento-Borne *et al.*, 2010). Over-containing salt cause soil-water unbalance especially in dry and semi-dry climate countries. Several factors contribute to the determination of salt levels in soil such as: over-cropping, irrigation and land management which are directly or indirectly affect the soil-bacterial communities. Electrical conductivity does not deliver any measurements of ions or salt compounds, however, it is an important indicator for the concentration of nitrates, potassium, sodium chloride, sulphate and ammonia (Fromme

et al., 2011). From the twelve soil-isolated samples, it was found that L (legumes) group has the highest capacity of EC with an average of 25.87 dSm^{-1}, moderate ECs were illustrated in R and G groups with an average of 14.87 and 12.65 dSm^{-1} respectively. The lowest level of EC was observed in C group 5.56 Sm^{-1}. Corresponding to the EC level, L group produced bacterial growth in the plates with the highest average number of *Azotobacter* (~ 97 CFU). One plate from the R group produced 370 and 70 CFUs with an *Azotobacter*-free plate at the concentration of 10 μl per plate. Grass group produced two *Azotobacter*-free plates with only 55 CFUs in the third plate. Finally, two plates from the C group propagated 94 and 108 CFUs with a bacterial free plate. These findings indirectly correlate between the EC and the soil *Azotobacter* abundance.

4. CONCLUSION

According to the current work results, isolation of *Azotobacter* spp. from variable ecological systems seems to be doable. The identification of *Azotobacter* species by traditional biochemical reaction is also achievable. However, it would be much better if bacterial identification carried out by using molecular tools. It appears that the *Azotobacter* availability, density, and biochemical activities such as; nitrogen cycle, the production of nitrous and other nitrogen oxides, respiration and chemical compounds decomposition cannot be determined by a sole environmental factor. The current results suggest that there are many environmental variable factors such as; soil acidity, soil texture, soil organic matter presence and electrical conductivity which contribute and interact with each other in a complex bio-net which in result regulate and determine the abundance of *Azotobacter* in soil.

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