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Study of saline effects in *Azospirillum* spp isolated from agricultural soils : south of Thi Qar governorate

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Abstract

Biofertilizers, containing microorganisms, colonize plant roots or interiors, increasing nutrient supply and stimulating growth. High salinity in agricultural soils reduces water absorption, growth rate, and photosynthetic activity, leading to physiological dehydration and growth inhibition. To develop biofertilizers, *Azospirillum* spp isolates were used, which are symbiotic nitrogen-fixing bacteria that promote plant growth, absorb minerals, and absorb water. The study aimed the impact of varying NaCl concentrations on the growth and effectiveness of Azospirillum spp isolates from five agricultural areas in the Fadliya district south of Thi Qar governorate. The isolates, all Gram-negative, were motile and responded to the requirements of biotin and pectin did not decompose during this study, except for the AZOST.3 isolate, they did not need decomposed biotin and pectin. The results of the current study showed the growth of *Azospirillum* spp at various levels of NaCl at a field capacity of 28% . All isolates recorded good growth at a minimum concentration of 3% NaCl, and growth gradually decreases with increasing salt concentration until reaching a concentration of 5% NaCl, which shows the extent of the portability of these isolates to grow at high concentrations of salt and its interference with humidity within the field capacity of 28%, as these isolates recorded more growth and tolerability of high salt concentrations compared to the low growth recorded by the AZO ST.1, AZO ST.2, and AZO ST.3 through a concentration of 4% NaCl at a field capacity of 14% .The study of the growth of *Azospirillum* spp at different pH levels also showed a variation in the growth intensity within the isolated strains of *Azospirillum*, as all five isolates recorded a gradual growth from the lowest level at pH 5.5 to pH 8.5 . It was also found that they can grow at high pH levels, which proves their potential as successful vaccines for cultivation in saline and alkaline soils.

Keywords: Biofertilizer, Azospirillum spp , Soil Salinity, Nitrogen-Fixing .

I. INTRODUCTION

The increasing global population necessitates increased agricultural productivity and food quality, but the excessive use of chemical fertilizers has led to pollution. To address this, researchers began searching for bacteria that contribute to biofertilization and nitrogen stabilization through coexistence with plants. Beijerinck first named the *Azotobacter* spp.,then Beijerinck (1925) renamed *Spirillum lipoferum*, a bacteria that developed a strong attachment to plant roots, in 1974 after its significance had declined for fifty years (Von and Döbereiner 1975). Interest in this bacterium was also sparked by its isolation from soil, grass roots, and cereal crops, as per (Döbereiner et al.,(1976). Tarand et al. (1978) were the first to propose *Azospirillum* spp, over the past 100 years, *Azospirillum* spp has seen significant taxonomy growth; some species are found in the Himalayan valley and Baiyang Lake. Advances in molecular biology have improved The classification of living organisms, with the C.C.Young group from National Chung Hsing University making significant contributions to this research (Young et al., 2008) and (Lin et al., 2016). They have discovered new species, redistributed others, and developed methodologies for identifying *Azospirillum* strains using technique polymerase chain reaction as per (Lin etal.2011). Their contributions have significantly contributed to the understanding of this type of bacteria. *Azospirillum* spp. a versatile genus, is primarily found in aquatic environments and has been isolated from soil. In its evolutionary process, it has moved from aquatic environments to terrestrial ones.. The genus is distributed worldwide, with different strains and species found in countries like Argentina, Brazil, Russia, Taiwan, Korea, China, Pakistan, and Iraq (Reis et al. 2015).

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Biofertilizer is a substance containing microorganisms that colonizes plant roots or interior, promoting growth by increasing primary nutrient supply. Nutrients are added through natural processes, such as fixation of nitrogen and the dissolution of phosphorus, and by producing chemicals that stimulate growth, it encourages growth of plants. (Vessey, 2003). *Azospirillum* spp is a type of bacteria that is used as one of the leading basic biofertilizers in nature (Roychowdhury et al., 2014). Which are Contribution in the process of nitrogen-fixing atmospheric to staple food crops like rice,
corn, sorghum, wheat, millet, and various elements (Bashan et al., 2004). intimately linked to the roots of several economically significant plants and grasses (Baldani et al., 1997). *Azospirillum* spp. produces growth hormones such as cytokinins, gibberellins, auxins,, polyamines, cadaverine, and siderophore and can solubilize inorganic phosphorus (Thuler et al., 2003). *Azospirillum* spp produces growth-regulating substances like polyamines, furthermore, cadaverine, which might be connected to stimulating root development (Niemi et al., 2002). Additionally dissolve phosphorus from inorganic sources. (Seshadri et al., 2000). Nitrogen-fixing bacteria with their coexisting and non-coexisting types are *Azotobacter* , *Azospirillum*, important bacteria in biofertilizing and highly efficient nitrogen-fixing free living organisms, as 100 nitrogen-fixing bacterial strains can be isolated from the rhizosphere, but *Azotobacter* and *Azospirillum* spp are no longer. one of the most efficient in terms of its ability to stabilize atmospheric nitrogen (Forlani et al., 1995). *Azospirillum* spp is narrowly found in the soil, and pollination of grain and fodder crops led to an extension of yields during the formation of basic substances to promote the development of plants. The difficulty with *Azospirillum* spp. exerting its advantageous effects lies in its capacity to colonize plant roots and remain viable in the presence of other local microbes residing in the root zone. As a result, t is necessary to conduct further studies of the relationships between *Azospirillum* species and plants, especially those that are their closest rivals. This obstacle can be overcome by producing vaccinations with more efficacy and vaccination techniques for planting. This entails drafting laws that set forth requirements for the trustworthy application of Azospirillum vaccines. For instance, the pollinators label ought to specify parameters including microorganism density at the time of production permissible contamination, pH, humidity, and salinity. Therefore, the study aimed to investigate how different salt concentrations and different pH levels affect the growth and effectiveness of isolated *Azospirillum* spp .

II. MATERIAL AND METHOD

Collection of agricultural soil samples

Soil samples were carefully collected from farms located in Fadliya district, south of Thi Qar governorate, at five random locations in the spring of 2023. Samples of soil were obtained between 0 and 30 cm below the surface of the soil. The samples were placed in clean plastic bags sterilized with alcohol and kept in the refrigerator until they were used to isolate *Azospirillum* spp, to test the chemical and physical properties of the soil, about 250 g was prepared, dried in the shade, and stored in a polythene bag. (Table 1).

the physical and chemical characteristics of the selected soil

The chemical and physical properties of soil samples, including pH, electrical conductivity (EC), organic carbon (OC), nitrogen (N), phosphorus (P), and potassium (K), were examined in the study according to Bremner (1965). and Soil Texture by Black (1965),.The samples were air dried, crushed, and ground using a pestle and mortar, then sieved through a 2 mm stainless steel sieve. The results provide insights into the quality of saline soil.

Isolation of Azospirillum

During the current study, the culture medium was used to isolate *Azospirillum* bacteria, according to (Baldani et al. 2014) and (Caceres ,1982). And used technique Hussein et al. (2015) to isolate *Azospirillum* spp, 0.1 ml of each specimen, dilution was added to screw-tubes containing NFB semi-solid medium, and the tubes were then incubated for 72 hours at 37 °C *Azospirillum* grew a thin, thick, white pellicle when it first emerged in the tubes. The pellicle was examined for G-negative, fibroid, and motile cells. After that, the pellicle was moved to a new, semi-solid NFB medium. (Figure 1), streaked on NFB medium plates, and solidified with 1.5% agar. For one week, the plates were incubated at 37°C.

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The isolates were then transferred to a solid malate medium and streaked on Red Congo medium for getting on pure colonies (Figure 2). The study clarified the biochemical, phenotypic, and microscopic characteristics of *Azospirillum* isolates, considering the diagnostic qualities of the genus *Azospirillum* and related species (Khammas et al. ,1989) and (Holt et al. (1994).

Sterilize the soil and prepare the bacterial vaccine.

For the purpose of eliminating the competition between the original microbiota and bacteria added to the soil during this study and the possibility of interfering with the final analyses of the influencing biological factors, the soil samples are sterilized for 1 hour by a sterilizer, then cooled and incubated at 30 C° for 24 hours, and then this process is repeated three times according to the method of Bashan et al. (1995). The required isolates are grown on a liquid nutrient agar medium by incubating at 30 C° for 24 hours. It is then taken by a loop carrier by adding sterile distilled water to it, according to Baron and Finegold (1990). Then 1 ml is taken to bottles containing sterile NFB medium, to which 0.1g NH_4Cl is added. And incubated at 30 C° for 72 hours according to Bashan et al. (1995).

The effect of salinity and moisture on the growth of *Azospirillum*

The weight of 100 g of prepared soil was placed in pots equipped with a field capacity of 28 ml of sterile distilled water, and then various concentrations of NaCL (3%,, 4%, and 5%) were added at three repetitions of each concentration. The second field capacity was 14 ml of sterile distilled water containing different concentrations of NaCL (3%, 4%, and 5%), with three repetitions for each concentration. Then these soils were inoculated with a volume of 1 ml of *Azospirillum* vaccine, the bacterial density of which was 10^{-4}, and then these soils were incubated at a degree of 37 C° for 15 days of incubation. The growth of *Azospirillum* was investigated in the treated soil model and replanted on the nutrient medium of the dens (Tensingh and Rajalakshmi, 2015).

Different pH values' effects on *Azospirillum* growth

The experiment was conducted using a liquid NFB medium with four different pH levels 5.5, 6.5, 7.5, and 8.5 with the addition of 1N of NaOH or HCL. The culture medium is then transferred to sterile and marked test tubes, and the tubes are inoculated with *Azospirillum* spp isolates separately. After incubation for 4 days, the observation of turbidity is evidence of the presence of growth (Usha and Kanimozhi, 2011).

III. RESULTS AND DISCUSSION

Soil samples were carefully collected from farms located in Fadliya district, south of Thi Qar governorate, from five random sites. The results of physical and chemical examinations of soil samples (Table 1) . The soil was loamy at all study sites, the pH value was 7.82–7.75, and the electrical conductivity (EC) recorded 3.18–3.24 ds/m^{-1}. The study showed N values of 40.81–37.6 mg/kg^{-1}, while P values were 7.82–7.55 mg/kg-1, and organic matter was 7.72–7.44 g/kg .Numerous elements, including soil types, climate, crop management techniques, and suitable pollination technologies, influence how bacteria behave in different environments (Hungria et al., 2013) .

Table 1 : certain chemical and physical properties of the soil used in the study.

Parameters	Station / Units	Soil St.1	Soil St.2	Soil St.3	Soil St.4	Soil St.5
pH	-	7.56	7.35	7.21	7.82	7.75
EC	ds.m-1	3.18	2.93	3.24	3.17	3.11
N	mg/kg-1	36.7	31.9	33.7	40.81	37.6
P	mg/kg-1	7.82	7.55	6.12	7.21	7.13
Organic matter	g/kg	7.44	7.72	6.81	6.11	7.2
Clay	g/kg	187	170	192	178	180
Silt	g/kg	510	529	535	512	518
Sand	g/kg	307	283	273	298	302

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Soil texture	%	loamy	loamy	loamy	loamy	loamy

A study isolated five strains from soil and studied their physical and biochemical properties, including interaction with Gram stain, cell shape, motility, oxidase and catalase tests, pectin hydrolysis, and biotin requirement, as shown in (Table 2).
The isolates during this study showed the shape of rod cells, all gram-negative and motile, which is one of the characteristic features of *Azospirillum* spp according to Tilik et al. (2010). *Azospirillum* isolates responded to biotin requirements. Pectin was not hydrolyzed during this study, except for AZO ST.3 isolation; it did not need biotin and was hydrolyzed by pectin, and this is consistent with (Tensingh Baliah and Rajalakshmi ,2015).

Table 2: Some morphological and biochemical properties of isolated *Azospirillum*.

Azospirillum isolates	Gram Staining	Bacterial Form	Motility	Oxidase Test	Catalase Test	Pectin Hydrolysis	Biotin Requirement
AZO ST.1	-	Rod	+	+	+	-	R
AZO ST.2	-	Rod	+	+	+	-	R
AZO ST.3	-	Rod	+	+	+	+	NR
AZO ST.4	-	Rod	+	+	+	-	R
AZO ST.5	-	Rod	+	+	+	-	R

- Negative / + Positive / R = Required / NR = Not Required

Figure: 2

Figure: 1

Figure 1: shows the white ring of Azospirillum growth on semi-solid NFB medium.
Figure 2: shows Azospirillum growth streaked on solid NFB medium plates

The results of the current study showed the growth of *Azospirillum* spp at different levels of NaCl at a field capacity of 28% (Table 3). All isolates recorded good growth at a minimum concentration of 3% NaCl, and growth gradually decreases with increasing salt concentration until reaching a concentration of 5% NaCl, which shows the extent of the portability of these isolates to grow at high salt concentrations and its interference with humidity within the field capacity of 28%, as these isolates recorded more growth and tolerability of high salt concentrations compared to the low growth recorded by the AZO

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ST.1, AZO ST.2, and AZO ST.3 through a concentration of 4% NaCl at a field capacity of 14% (Table 4). This is consistent with what Yogaraj et al. (2021) and Usha and Kanimozhi (2011).

Table 3: description of the growth of *Azospirillum* isolates at different concentrations of NaCl at a field capacity of 28 ml.

Azospirillum isolates	Bacterial density	*Azospirillum* growth at different NaCl concentrations at field capacity of 28 ml		
		NaCl 3 %	NaCl 4 %	NaCl 5 %
AZO ST.1	$10*9^{-4}$	++	++	+
AZO ST.2	$10*11^{-4}$	+++	++	+
AZO ST.3	$10*13^{-4}$	+++	++	+
AZO ST.4	$10*10^{-4}$	++	++	+
AZO ST.5	$10*9^{-4}$	++	+	+

+ = good growth / ++ = very good growth / +++ = Excellent growth

Table 4: Description of the growth of *Azospirillum* isolates at different concentrations of NaCl at a field capacity of 14 ml.

Azospirillum isolates	Bacterial density	*Azospirillum* growth at different NaCl concentrations at field capacity of 14 ml		
		NaCl 3 %	NaCl 4 %	NaCl 5 %
AZO ST.1	$8*10^{-4}$	++	+	+
AZO ST.2	$11*10^{-4}$	+++	+	+
AZO ST.3	$12*10^{-4}$	+++	+	+
AZO ST.4	$9*10^{-4}$	++	++	+
AZO ST.5	$9*10^{-4}$	++	+	+

+ = good growth / ++ = very good growth / +++ = Excellent growth

The study of the growth of *Azospirillum* spp at different pH levels also showed a variation in the growth intensity within the isolated strains of *Azospirillum*, as all five isolates recorded a gradual growth from the lowest level at pH 5.5 to pH 8.5 (Table 5). This shows that *Azospirillum* spp can grow at a high pH level. This is consistent with Yogaraj et al. (2021).

Table 5: Description of *Azospirillum* grown at different pH values

Azospirillum isolates	Growth of *Azospirillum* in a different pH values			
	pH 5.5	pH 6.5	pH 7.5	pH 8.5
AZO ST.1	+	++	+++	+
AZO ST.2	+	++	+++	+
AZO ST.3	+	+++	+++	+
AZO ST.4	+	++	+++	+
AZO ST.5	+	+++	++	+

+= good growth / ++ = very good growth / +++ = Excellent growth

IV. CONCLUSIONS

Despite extensive scientific data on *Azospirillum* spp , our understanding of their importance in agricultural and environmental technologies remains limited. *Azospirillum* spp is a bacterial genera that efficiently and safely fixes nitrogen. Further research should focus on basic studies, field and laboratory experiments, and new scientific approaches. Using different genome sequences and new scientific approaches will provide more insight and improve *Azospirillum* spp biotechnology's use in agriculture and addressing environmental pollution problems . The study investigated the impact of salt

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concentrations and moisture interference on the growth and activity of *Azospirillum* spp in agricultural soils. Results showed that all moisture parameters and three salt concentrations affected bacteria growth differently, indicating they can tolerate over 5% soil salinity. However, high pH levels *Azospirillum* spp susceptibility to growth.

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