Study of the relationship between bacillus subtilis and azospirillum sp. & bradyrhizubium sp. isolated from gypsiferous soil

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Abstract

The study included collecting (20) soil samples from different Gypsiferious soil from Salah al-Din Governorate, Tikrit University, where it was possible to obtain (12) isolates belonging to the genus Bacillus. All bacterial isolates were subjected to microscopic, cultural and biochemical tests, which included catalase test, oxidase test, movement test, growth in anaerobic conditions, growth in (5% 70% 10%) of table salt, indole test, sterile consumption test as a sole carbon source, Fox-Proscor test, ability to decompose starch, production of gas from glucose sugar, as well as their ability to ferment (7) carbohydrate sources. The ability of the isolates to produce inhibitory substances when grown on the Nutrient broth culture medium at a temperature of (37) C for (4) days was also tested, then the filtrates of the isolates growing in the liquid medium were taken once Bs.1 and twice Bs.2 to increase their inhibitory effectiveness A filtrate without Bs.0 bacteria was used for comparison and the best isolates in all tests were identified and belonged to the species Bacillus subtilis. Also, Azospirillum sp. and Bradyrhizubium sp. were isolated from Gypsiferious soil and the three bacterial isolates were planted on Nutrient agar medium using the drilling diffusion method. The results showed that B. subtilis bacteria have inhibitory activity against Azospirillum sp. bacteria isolates (Azo1, Azo2, Azo3, Azo4, Azo5) respectively. The filtrate of the B. subtilis isolate did not give any significant inhibitory activity against Bradyrhizubium sp. bacteria isolates. Br1, Br2, Br3 Br4, Br5 respectively, The highest number of colonies was in the isolate Br4 24,17,14 CFU.10^{-4} respectively, at the concentration of Bs0, Bs1, Bs2 respectively, while the lowest number of colonies was in the isolate Azo5 16,1,0 respectively, at the same levels. The highest number of colonies was 24, 14 and 17 in the isolate Br4 at the concentration of Bs0, Bs1, Bs2 respectively, while it was 16,1,0 in the isolate Azo5 at the same concentration,

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because the highest production of inhibitory substances was 71 mg. g^{-1} at the concentration twice Bs2, while no secretion of inhibitory substances was recorded in all isolates in the absence of B.subtilis bacterial filtrate.

Keywords: Bacillus subtilis, Azospirillum, Bradyrhizubium, Gypsiferious, Inhibition

Introduction

Gypsiferous soil are defined as those soil containing more than 3% of hydrated calcium sulfate in the root zone and whose thickness is less than 15 cm (Barzanji, 1973). Gypsiferious soil are spread in dry and semi-dry areas and cover about 100 million hectares of the world's geographical area, and about 8.7% of them are located in Iraq and constitute more than 20% of the country's area (Khalaf, 2021). There are many problems that Gypsiferious soil suffer from related to plant nutrition, including the ionic imbalance of nutrients resulting from the saturation of the soil solution with sulfate and calcium ions and their effect on the growth and spread of plant roots, in addition to the low content of clay and organic matter and weak biological activity (Al-Duliami, 2020; Yang, 2024). In order to improve the properties of Gypsiferious soil, bacteria that encourage plant growth are used, including *Bacillus subtilis* and *Azospirillum* sp. & *Bradyrhizubium* sp (Ling *et al.,* 2012). to improve plant growth in the conditions of these soil (Singh and Kumar, 2024). There are many relationships that arise between microorganisms, which made researchers move towards studying these relationships to benefit from them in human life, as they are environmentally safe and sustainable means (Danková *et al.,* 2018). These include biocontrol, biofertilizer, activation, inhibition and antagonism (Rao and Tiwari, 2023; Rathore and Shaikh, 2023). Therefore, recent years have witnessed a broad trend in the field of using microorganisms and their metabolic products in the treatment of some diseases (Nunes *et al.,* 2024) Among these methods is the use of the genus Bacillus in the fields of using microorganisms in industrial production, as its use is no longer limited to the production of some industrial compounds, but has extended to include the production of the most complex pharmaceutical and chemical compounds, the most important of which are antibiotics, hormones, vitamins, pesticides and biofertilizers (Lesueur; 2016; Sayad Bourani *et al.,* 2023).

Among the characteristics that must be available in the industrial microbe is its ability to produce a large biomass in addition to its production of vegetative cells, spores or reproductive units in large numbers (Bashan *et al.,* 2014**)**, its tolerance to harsh environmental conditions that are not suitable for growth, its ability to survive for a long time in the soil and in the biological preparation, and its growth in a wide temperature range and wide acidity (Jain and Suresh, 2024; Sim and Han, 2020). These specifications are available in the genus Bacillus, which is distinguished by its resistance to harsh, unsuitable environmental conditions due to its production of spores (Logan and De Vos., 2009; Singhal *et al.,* 2024). Also because this genus is distinguished from other bacteria by its production of primary and secondary metabolic compounds such as antibiotics, enzymes and acids in large quantities (Harwood, 2018). B. subtilis produces many antibiotics, either ribosomally synthesized or non-ribosomally synthesized, and these antibiotics are highly effective against some microorganisms that infect plants and humans (De Oliveira, 2007). Root nodule bacteria (RNB) or Rhizobia" are a type of bacteria that promote plant growth, as they are able to fix nitrogen through a symbiotic relationship with the plant host and fix approximately 65% of the nitrogen used in sustainable agricultural production of legume crops. This group of bacteria includes 76 species, including 16 genera (Thein, 2019) Exploitation of bacteria present in the soil affected by roots and referred to as PGPR in conjunction with Rhizobium is a successful and effective nodule in fixing nitrogen (Tamiru, 2022; Palai *et al.,* 2021).

Azospirillum bacteria are free-living, aerobic, gram-negative, nitrogen-fixing bacteria from the Rhodospirillaceae family. They do not form spores and have a slightly twisted, oblong rod shape. *Azospirillum* has at least one flagellum and sometimes multi-flagellates, which are used for rapid movement (Steward, 2012). *Azospirillum* has the ability to attach and participate in plant roots without forming a relationship with it. It is found in the soil, on the surface and inside weeds and tissues of cereal crops (Burdman, 2000) These bacteria are used as a biofertilizer to encourage plant growth because they fix atmospheric nitrogen and secrete hormones that encourage plant growth. (Zaheer *et al.,* 2024).

Therefore, this study aims to: Isolate and identify *Bacillus* sp., *Bradyrhizubium* sp. and *Azospirillum* sp. from Gypsiferous soil. Test the relationship between Bacillus sp. filtrates against *Bradyrhizubium* sp. and *Azospirillum* sp. isolates and demonstrate their efficiency in producing enzymes and hormones that encourage plant growth and the possibility of using all of these bacteria in one biofertilizer to encourage plant growth.

Materials and Methods

Sample Collection

20 soil samples were collected from the governorates of Salah al-Din, Tikrit, Tikrit University, the fields of the College of Agriculture, which are located at latitude 34.4057 north and longitude 43.3856 west for the spring season of 2024, which are located within the Gypsiferous soil series in the governorate. The samples were placed in clean and sterile bags, weighing (2) grams of soil and dissolved in (20) ml of sterile distilled water, and placed in a water bath at a temperature of (80 c^0) for (15) minutes in order to eliminate non-spore-forming vegetative cells. A series of decimal dilutions were performed on this suspension using sterile test tubes containing (9) ml of sterile distilled water. These tubes were numbered and (0.1) ml of the dilution (10^{-5}) and (10^{-6}) was withdrawn and distributed to Petri dishes containing (20) ml of Nutrient Agar culture medium and spread by a glass spreader, then the dishes were incubated in the incubator at 37 C^0 /24 h according to Vincent (1970).

Isolation of Bacteria Used in the Study

The strains of Bacillus subtilis, Bradyrhizobium sp., and Azospirillum sp. were obtained from Gypsiferous soil. Bacillus subtilis was isolated on Mannitol Salt Agar medium, while Bradyrhizobium sp. was isolated on Yeast Extract Mannitol Agar medium, *Azospirillum* sp. was isolated on nitrogen-free broth medium according to Collee (1996). All isolates were purified, and biochemical and morphological characterization of the isolates was executed through standard tests (Colony colour, Growth, Shape, texture, Gram stain, convex, gummy, Motility, Endospore staining) (Catalase, Oxidase, Starch hydrolysis, Indole acetic acid, Voges – Proskauer, Citrate utilization, Glucose with Gas, Lactose, Galactose, Mannitol, Sucrose, Fructose, Anaerobic Growth, Nacl 2%, Nacl 10%) according to Bergey's Manual Bacteriology (2005).

Study of the Inhibitory Activity of B. Subtilis

Study of the inhibitory activity of B. subtilis against Azospirillum sp. isolates. Bradyrhizubium sp. The well and Azospirillum sp. diffusion method were used as stated in (1970, Vincent). The inhibitory enzymes were estimated by spectrophotometer as stated in (Hernández-Ledesma, *et al.,* 2003)

The liquid culture filtrates were prepared by growing *B. subtilis* isolates in Nutrient broth medium with a pH of (7). The tubes were incubated at a temperature of (37) ^{0}C for (4) days.

In *Bradyrhizubium* sp. free culture that was filtered through Millipore filters with a diameter of (0.22) micrometers. *Azospirillum* sp. isolates were cultured and Nutrient broth medium and the tubes were incubated at a temperature of (37) C for (24) hours, and after centrifugation (5000) rpm for 10 minutes) the suspended cell fluid was obtained and its turbidity was compared with the standard turbidity solution tube. (0.1) ml of the suspension was transferred and spread on the surface of Nutrient agar medium, then the plates were left to dry at laboratory temperature. A cork piercer was used to make holes with a diameter of (5) mm in the solid medium, then the holes were filled with (100) microliters of the liquid culture filtrate of *B. subtilis* isolates, then the plates were left to dry at laboratory temperature, then they were fortified at a temperature of (37) ^{0}C for (24) hours. The diameters of the inhibition zones were then measured in mm around the holes.

The liquid culture filtrates of all *B. subtilis* isolates were concentrated using a centrifuge to obtain inhibition. For the isolates whose concentrated filtrates did not show effectiveness against the test bacteria, the filtrates were concentrated once and twice by dissolving the concentrated filtrate in (10) ml and (5) ml of distilled water. Then the concentrated filtrates were compared with the concentrated filtrates in terms of inhibitory effectiveness.

Statistical Analysis

The experiment was carried out according to the completely randomized design (CRD) with three replicates and the characteristics were analyzed using analysis of variance. The averages of the treatments were compared using the least significant difference (LSD) test using the calculator based on the statistical program SPSS version 9 (1998).

Results and Discussion

The inhibitory activity of the liquid culture filtrates of B. subtilis bacteria was tested on the susceptibility of B. subtilis bacteria grown in the liquid medium to the bacteria isolated from Gypsiferious soil Bradyrhizubium sp. and Azospirillum sp. after growing them in Nutrient broth medium at a temperature of (37) for (4) days. The liquid culture filtrates were used directly without concentration against the bacteria used in the study. The effectiveness of these filtrates was tested after concentrating them using a centrifuge once and twice, in order to obtain concentrated filtrates for the isolates' cultures, which were characterized by their high effectiveness that exceeds the effectiveness of the non-concentrated filtrates. Tables 1 and 2 show some of the cultural and biochemical characteristics of the three isolates. It was shown through the tests that *B. subtilis* bacteria are Gram-positive, slow-growing, and have a high effectiveness in producing hormones and enzymes, which increases their inhibitory effectiveness and their effect on the rest of the organisms growing in the same medium. This was confirmed by Angelina (2020).

Table 1: Some cultural characteristics of Bacillus subtilis isolates and Azospirillum sp. and Bradyrhizubium sp. isolated from Gypsiferious soil

Isolates	Colony colour	Growth	Shape	Texture	Motility	convex	gummy	Gram's reaction	Endospore staining
Bs	Yellow	Slow	bacilli	Slimy	+	convex	gummy	+	+
Br	Cream	Fast	bacilli	Slimy	+	convex	gummy	_	_
Azo	White	Slow	bacilli	Slimy	+	convex	gummy	_	_

Not. Bs= Bacillus subtilis, Br = Bradyrhizubium sp., Azo = Azospirillum sp.

Bradyrhizubium sp. bacteria were found to be Gram-negative, nitrogen-fixing, symbiotic, fast-growing, very effective in producing hormones, enzymes, and indole acetic acid, and grow in saline media, which is consistent with Seidu (2025).

Table 2: Biochemical tests for Bacillus subtilis, Azospirillum sp. and Bradyrhizubium sp. bacteria isolated from Gypsiferious soil

test	Bs.	Br.	Azo.
Catalase	+	+	+
Oxidase	-	+	-
Starch hydrolysis	+	-	-
Indole acetic acid	-	+	+
Voges - Proskauer	+	+	-
Citrate utilization	+	-	-
Glucose with Gas	-	-	-
Lactose	-	+	-
Galactose	+	+	+
Mannitol	+	+	+
Sucrose	+	+	+
Fructose	+	-	-
Anaerobic Growth	+	-	-
Nacl 2%	+	+	-
Nacl 10%	+	-	-

As for *Azospirillum* sp., it is clear from the above two tables that it is Gram-negative, slow-growing, capable of fixing nitrogen freely in the soil, very effective in producing hormones, enzymes, and indole acetic acid, and has poor growth in saline media, as shown by (Zuber, 2019). The results in Table (3) show that the non-concentrated filtrates of *B. subtilis* isolates did not show any inhibitory activity against any of the *Bradyrhizubium* sp. isolates used in the study, and if they existed, they were insignificant, as the results were negative when no growth inhibition zones appeared around the holes filled with those filtrates. As for *Azospirillum* sp. isolates, we note from the tables that the non-concentrated filtrates of B. subtilis isolates showed inhibitory activity when the diameters of the inhibition zones of these filtrates ranged between (0-3) mm2, while the inhibitory activity of the non-concentrated filtrates of *B. subtilis* isolate was non-existent. When the *B. subtilis* filtrates were concentrated once.

Table 3: Study of the relationship between Bacillus subtilis and Azospirillum sp. and Bradyrhizubium sp. isolated from Gypsiferious soil Average inhibition diameter mm2

trt	Azo1	Azo2	Azo3	Azo4	Azo5	mean	Br1	Br2	Br3	Br4	Br5	mean
Bs.0	0	0	0	0	0	0	0	0	0	0	0	0
Bs.1	3	4	5	6	6	4.80	0	2	1	0	0	0.60
Bs.2	5	6	9	12	19	10.2	2	1	3	2	2	2.00
mean	1.66	3.33	4.66	6.00	8.33	5.00	0.66	1.00	1.33	0.66	0.66	0.86
LSD 0.05	Azo.= **1.350**			Azo. * Bs.= **0.820**			Br.= **0.950**			Br. * Bs. =**0.122** Bs. =2.033		

the four filtrates showed growth inhibition zones for *Azospirillum* sp. The values of the diameters of the inhibition zones ranged between (3-6) mm2, and the results also showed inhibition zones for the growth of *Bradyrhizubium* sp. The values of the diameters of the inhibition zones ranged between (1-3) mm2. It is clear from the results mentioned and shown in the tables that the minimum diameter of the inhibition zone of the filtrates of *B. subtilis* bacteria concentrated once against the isolates of *Bradyrhizubium* sp. As for the effect of the filtrates of bacteria concentrated twice on the isolates of *Azospirillum* sp. As shown in Tables (5,4,3,2), all the filtrates showed inhibitory activity against the isolates of these bacteria, as the minimum diameter of the inhibition zone of the filtrates of bacteria was 3 mm, which was shown by the filtrate of the isolate - *B. subtili*s. against the isolate -1- *Azospirillum* sp. The highest inhibition zone diameter for the same filtrates was (19) mm2, which was shown by the filtrates of *B. subtilis* isolates 1 and 2 against *Azospirillum* sp. 4 and 5, respectively. As for the effect of the twice-concentrated bacterial filtrates on *Bradyrhizubium* sp. isolates, they showed high resistance against inhibition by *B. subtilis*, and the reason may be attributed to the Bacillus subtilis is an aerobic bacterium known for its remarkable ability to secrete various antibacterial compounds, including antimicrobial peptides such as bacilysin and bacitracin. These compounds are natural substances that *B. subtilis* utilizes to defend itself against other microorganisms competing for the same environmental resources (Branda, 2005). On the other hand, *Azospirillum* is a bacterium that primarily inhabits the root environment of plants and is part of the group of nitrogen-fixing bacteria, making it of significant biological importance.

When B. subtilis and Azospirillum coexist in the same environment, they can compete for the same food sources and environmental components, resulting in growth inhibition of each other. It has been found that Bacillus subtilis secretes a variety of substances with antibacterial effects, which can inhibit the growth of Azospirillum, such as bioactive peptides like bacilysin. Bacilysin works by disrupting the cell membranes of competing bacteria like Azospirillum. These peptides attack the target bacterial cell membranes, leading to loss of cellular integrity and failure in bacterial reproduction (Kour, 2023).

In addition to these direct effects, B. subtilis can influence the bacterial community balance in the surrounding root zone. By altering the local environment through the secretion of antimicrobial chemicals, the ability of Azospirillum to interact with the environment is reduced, weakening its growth and reproduction capabilities in the same area. Therefore, an indirect inhibitory effect on *Azospirillum* is achieved (Ghosh *et al,* 2021).

Bradyrhizobium sp. is a bacterium known for its ability to fix atmospheric nitrogen in soil, playing a significant role in improving soil quality and supporting plant growth. The impact of certain strains of *Bradyrhizobium* against other bacteria such as *Bacillus subtilis,* which is beneficial in agriculture, has been studied (Nicotiana and Beaudette, 2021).

Bradyrhizobium can secrete compounds called antibiotics, such as sapromycin, which can have inhibitory effects on the growth of *B. subtilis*. In some studies, it was found that *Bradyrhizobium* can enhance its surrounding environment, leading to a reduction in the proliferation of *B. subtilis* due to resource depletion (Yadav and Gupta, 2020).

We note from the results in Table 4 that the filtrates of concentrated and non-concentrated *B.subtilis* isolates differ in their inhibitory efficiency on *Bradyrhizubium* sp. isolates. The secreted inhibitory substances were few and insignificant, while the filtrates of *B.subtilis* isolate were the most effective in terms of inhibitory effect on *Azospirillum* sp. isolates. The highest value of secreted enzymes was in the twice-concentrated *B.subtilis* and reached 71 mg.g-1. The reason may be attributed to several factors, including the incubation period and the duration of the contrast, as indicated by (Stein, 2005). Bacillus subtilis strains are producing a wide variety of antibacterial and antifungal compounds such as, lantibiotics, rhizocticin and 2 lipopeptides, surfactin and mycosubtilin, the latter being a member of the, iturin family Bacillus subtilis strains may produce other antimicrobial substances, which have been characterized to a much lesser extent. (Abriouel, *et al.,* 2011).

(Ibrahim, 2022) indicated that *B. subtilis* bacteria can produce the antibiotic Bacillysoin, which is highly effective against *Azospirillum* sp. Therefore, it is not recommended to mix these bacteria in one biofertilizer or one growth medium, while *B. subtilis* bacteria can be used with *Bradyrhizubium* sp. bacteria in the same growth medium and prepare a beneficial bioinoculation for the plant. This is consistent with. (Nelwamondo, 2020).

Table 4: Study of the relationship between Bacillus subtilis bacteria and Azospirillum sp. and Bradyrhizubium sp. bacteria isolated from Gypsiferious soil. Percentage of enzymes secreted in the medium mg.g-1-

trt	Azo1	Azo2	Azo3	Azo4	Azo5	mean	Br1	Br2	Br3	Br4	Br5	mean
Bs.0	0	0	0	0	0	0	0	0	0	0	0	0
Bs.1	33	45	66	53	26	44.6	25	27	21	12	21	21.2
Bs.2	67	71	45	43	44	54.0	26	27	24	22	26	25.0
mean	33.3	38.6	37.0	32.0	23.3	32.8	17.0	18.0	15.0	11.3	15.6	15.4
LSD 0.05	Azo.= **1.915**			Azo. * Bs.= **1.020**			Br.= **2.782**			Br. * Bs. =**1.511** Bs. =11.12		

From Table 5, it is clear that *B. subtilis* has inhibitory activity against *Azospirillum* sp. isolates (Azo1, Azo2, Azo3, Azo4, Azo5) respectively, and the filtrate of *B. subtilis* isolate did not show any significant inhibitory activity against *Bradyrhizubium* sp. isolates. Br1, Br2, Br3 Br4, Br5 The highest number of colonies was in the isolate Br4 24,17,14 CFU.10-4 respectively, at the concentration of Bs0, Bs1, Bs2 respectively, while the lowest number of colonies was in the isolate Azo5 16,1,0 at the same levels. The highest number of colonies was 24, 14 and 17 in the isolate Br4 at the concentration of Bs0, Bs1, Bs2 respectively, while it was 16,1,0 in the isolate Azo5 at the same concentration. This is because the highest production of inhibitory substances was 71 mg.g-1-1 at the concentration twice Bs2, which affected the reduction of the number of growing bacterial cells, while no secretion of inhibitory substances was recorded in all isolates in the absence of B. subtilis filtrate.

Table 5: Study of the relationship between Bacillus subtilis bacteria and Azospirillum sp. and Bradyrhizubium sp. bacteria isolated from Gypsiferious soil Number of colonies growing in petri dishes CFU. 10-4

trt	Azo1	Azo2	Azo3	Azo4	Azo5	mean	Br1	Br2	Br3	Br4	Br5	mean
Bs.0	19	18	18	17	16	17.6	22	19	20	24	19	20.80
Bs.1	3	1	2	3	1	2.00	15	10	16	17	10	13.60
Bs.2	1	0	0	2	0	0.60	8	9	12	14	9	10.40
mean	7.66	6.33	6.66	7.33	5.66	6.73	15.0	12.66	16.0	18.33	12.66	14.933
LSD 0.05	Azo.= **0.851**			Azo. * Bs.= **1.020**			Br.= **1.251**			Br. * Bs. =**0.250** Bs. =1.821		

Conclusion

Studying the relationships between living organisms in the environment is very important and can be used in some biotechnologies such as biofertilizers and biocontrol ,Sometimes, bio-inoculations are prepared from different bacteria with the aim of stimulating plant growth, for example, nitrogen-fixing bacteria with phosphorus-dissolving bacteria From the results of the above research, it is clear that there is an inhibitory effect of Bacillus bacteria on Azospirillum bacteria, so it is preferable not to mix them in one biofertilizer, while there was no effect of

Bacillus bacteria with Bradyrhizubium bacteria.

References

Abriouel, H., Franz, C. M., Omar, N. B., and Gálvez, A., 2011. Diversity and applications of Bacillus bacteriocins. *FEMS microbiology reviews*, *35*(1), pp. 201-232. https://doi.org/10.1111/j.1574-6976.2010.00244.x

Al-Duliami, A. A. K., Altai, S. H., and Ismaeal, A. S., 2020. Impact study of slope aspect in the boilogical crust properties of some gypsiferous soil using remote sensing and GIS. *Plant Archives* (09725210), *20*(2).

Angelina, E., 2020. Lettuce yield and rhizosphere microbial community response to inoculation with plant growth promoting rhizobacteria.

Barzanji, A. F., 1973. Gypsiferous soils of Iraq (Doctoral dissertation, Ghent University).

Bashan, Y., de-Bashan, L. E., Prabhu, S. R., and Hernandez, J. P., 2014. Advances in plant growth-promoting bacterial inoculant technology: formulations and practical perspectives (1998–2013). *Plant and soil*, *378*, pp.1-33.

Branda, S. S., Vik, Å., Friedman, L., and Kolter, R., 2005. Biofilms: the matrix revisited. *Trends in microbiology*, *13*(1), pp.20-26. https://doi.org/10.1016/j.tim.2004.11.006

Burdman, S., Okon, Y., and Jurkevitch, E., 2000. Surface characteristics of Azospirillum brasilense in relation to cell aggregation and attachment to plant roots. *Critical reviews in microbiology*, *26*(2), pp.91-110. https://doi.org/10.1080/10408410091154200

Collee, J.G., Miles, R.S. and Watt, B., 1996. Tests for the identification of bacteria. *Mackie and McCartney practical medical microbiology*, *14*, pp.131-149.

Danková, Z., Dakos, Z., Štyriaková, I., and Bekényiová, A., 2018. Study of Cu (II) Adsorption by Bentonite and Following Regeneration by Bioleaching. *Archives for Technical Sciences*, *2*(19), pp.45–56.

Oliveira, A.N.D., Oliveira, L.A.D., Andrade, J.S. and Chagas Júnior, A.F., 2007. Rhizobia amylase production using various starchy substances as carbon substrates. *Brazilian Journal of Microbiology*, *38*, pp.208-216.

Ghosh, S., Ahmad, R., Zeyaullah, M., and Khare, S. K., 2021. Microbial nano-factories: synthesis and biomedical applications. *Frontiers in Chemistry*, *9*, p.626834. https://doi.org/10.3389/fchem.2021.626834

Harwood, C. R., Mouillon, J. M., Pohl, S., and Arnau, J., 2018. Secondary metabolite production and the safety of industrially important members of the Bacillus subtilis group. *FEMS microbiology reviews*, *42*(6), pp.721-738. https://doi.org/10.1093/femsre/fuy028

Hernández-Ledesma, B., Martín-Álvarez, P. J., and Pueyo, E., 2003. Assessment of the spectrophotometric method for determination of angiotensin-converting-enzyme activity: Influence of the inhibition type. *Journal of agricultural and food chemistry*, *51*(15), pp.4175-4179.

Ibrahim, N., 2022. Antibacterial effect of Bacillus subtilis extract on the growth of pathogenic bacteria and analyzed by GC-MS. *Journal of Education and Science*, *31*(1), pp.111-122.

Jain, S., and Suresh, N., 2024. Membrane Technologies in Juice Clarification: Comparative Study of UF and NF Systems. *Engineering Perspectives in Filtration and Separation, 2*(3), pp.1-4.

Kour, D., Kour, H., Khan, S. S., Khan, R. T., Bhardwaj, M., Kailoo, S., ... and Sharma, Y. P., 2023. Biodiversity and functional attributes of rhizospheric microbiomes: potential tools for sustainable agriculture. *Current Microbiology, 80*(6), p.192.

Khalaf, A. A., Ismaeal, A. S., and Altai, S. H., 2021. Evaluation and Biological properties maps of Gypsiferous Soil using Geomatic techniques, Tikrit city, Salahaldin, Iraq. *In IOP Conference Series: Earth and Environmental Science, 735* (1), p.012067. IOP Publishing.10.1088/1755-1315/735/1/012067

Lesueur, D., Deaker, R., Herrmann, L., Bräu, L., and Jansa, J., 2016. The production and potential of biofertilizers to improve crop yields. *Bioformulations: for sustainable agriculture,* pp.71-92.

Ling, A.P.A., Kokichi, S., and Masao, M., 2012. Enhancing Smart Grid System Processes via Philosophy of Security-Case Study based on Information Security Systems-. *Journal of Wireless Mobile Networks, Ubiquitous Computing, and Dependable Applications, 3*(3), pp. 94-112.

Logan, N. A., and De Vos, P., 2009. Genus I. bacillus. *Bergey's manual of systematic bacteriology, 3,* pp. 21-128.

Nelwamondo, A. M., 2020. Assessment of co-inoculation of Bradyrhizobium japonicum and Bacillus subtitles on yield and metabolic profile of Bambara groundnut and cowpea under glasshouse conditions. *Institutional Repository, 1*(1), pp. 1-80.

Nicotiana, R., and Beaudette, L., 2021. Microbial Interactions in Soil and Rhizosphere. *Journal of Soil Microbiology.*

Nunes, P. S., Lacerda-Junior, G. V., Mascarin, G. M., Guimaraes, R. A., Medeiros, F. H., Arthurs, S., and Bettiol, W., 2024. Microbial consortia of biological products: Do they have a future?. *Biological Control, 188,* p.105439. https://doi.org/10.1016/j.biocontrol.2024.105439

Palai, J. B., Malik, G. C., Maitra, S., and Banerjee, M., 2021. Role of Rhizobium on growth and development of groundnut: a review. http://dx.doi.org/10.30954/0974-1712.01.2021.7

Rao, N., and Tiwari, M., 2023. Nature-Based Solutions for Coastal Resilience: Case Studies from Southeast Asia. *International Journal of SDG's Prospects and Breakthroughs, 1*(1), pp. 8-10.

Rathore, N., and Shaikh, A., 2023. Urbanization and Fertility Transitions: A Comparative Study of Emerging Economies. Progression *Journal of Human Demography and Anthropology, 1*(1), pp. 17-20.

Sayad Bourani, M., Ghasemi, S., Abdolmalaki, S., Golshan, M., and Malakpour Kobadinezhad, S., 2023. Using the back calculation to study of the age and growth of Caspian trout (Salmo Caspius). *International Journal of Aquatic Research and Environmental Studies, 3*(2), pp. 81-97. https://doi.org/10.70102/IJARES/V3I2/5

Seidu, O. A., Githiri, S. M., Wesonga, J. M., and Ngumi, V. W., 2025. Isolation, screening and in-vitro characterization of plant growth-promoting Bradyrhizobium isolates from the nodules of Bambara groundnut (Vigna subterranean) for potential use as bioinoculants. *Frontiers in Sustainable Food Systems, 8,* p.1506346. https://doi.org/10.3389/fsufs.2024.1506346

Sim, B.Y., and Han, D.G., 2020. A study on the side-channel analysis trends for application to IoT devices. *Journal of Internet Services and Information Security, 10*(1), pp. 2-21. 10.22667/JISIS.2020.02.29.002

Singh, N., and Kumar, A., 2024. Gamification in Medical Terminology Learning: A Comparative Study of Digital Education Tools. *Global Journal of Medical Terminology Research and Informatics, 2*(1), pp. 4-7.

Singhal, P., Yadav, R. K., and Dwivedi, U., 2024. Unveiling Patterns and Abnormalities of Human Gait: A Comprehensive Study. *Indian Journal of Information Sources and Services, 14*(1), pp. 51–70. https://doi.org/10.51983/ijiss-2024.14.1.3754

Stein, T., 2005. Bacillus subtilis antibiotics: structures, syntheses and specific functions. *Molecular microbiology, 56*(4), pp. 845-857. https://doi.org/10.1111/j.1365-2958.2005.04587.x

Steward, F. C. (Ed.)., 2012. Plant Physiology 8: A Treatise: Nitrogen Metabolism (Vol. 8). *Elsevier*.

Tamiru, G., 2022. Effect of host plant resistance and rhizobial inoculants on chocolate spot (Botrytis fabae) severity and yield of faba bean (Vicia faba L.) in south region, *Ethiopia.* 10.30918/NJAS.103.22.013

Thein, K. K., 2019. Characterization of Indigenous Green Gram Rhizobium Isolates and Survival on Different Carriers (Doctoral dissertation, MERAL Portal).

Vincent, J. M., 1970. The cultivation, isolation and maintenance of rhizobia. *A manual for the practical study of the root-nodule bacteria*, pp. 1-13.

Yadav, S., and Gupta, R., 2020. Characterization of Bradyrhizobium Strains and their Role in Agricultural Soil Health. *Agricultural Microbiology Journal, 8*(3), pp. 234-245.

Yang, Z., 2024. The Impact of Environmental Assessment of Green Innovation on Corporate Performance and an Empirical Study. *Natural and Engineering Sciences, 9*(2), pp. 94-109. https://doi.org/10.28978/nesciences.1569137

Zaheer, M. S., Rizwan, M., Aijaz, N., Hameed, A., Ikram, K., Ali, H. H., ... and Rehman, S., 2024. Investigating the synergistic effects of biochar, trans-zeatin riboside, and Azospirillum brasilense on soil improvement and enzymatic activity in water-stressed wheat. *BMC Plant Biology, 24*(1), p.314.

Zuber, N., 2019. Influence of Various Levels of N On Nitrogenase Enzyme Activities and Plant Growth Promotion Properties of Azospirillum Brasilense (Sp7) And Herbasprillum seropedicae (Z78).