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Original Research Article

Biochemical and molecular diagnosis of bacteria associated with Cladophora algae and their effect on some growth characteristics of wheat plants

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Abstract: A sample of Cladophora algae was collected from te Euphrates River, the Atshan River,and the Third River in Al-Muthanna, Iraq, for molecular diagnosis of the bacteria associated with these algae. The results showed the presence of five isolates of bacterial: Bacillus cereus, Bacillus paramycoides, Streptomyces griseolus, Klebsiella aerogenes, and Citrobacter freundi.Differential and biochemical tests confirmed the in vitro diagnosis using molecular diagnostics. Five laboratory experiments were also conducted to determine the characteristics of the isolates in terms of ammonia production, sideophore production, and hydrogen cyanide production. An experiment was conducted to investigate the ability of bacterial isolates to form biofilms for use in bio fertilization. The results showed varying production capacities. All isolates exhibited a high capacity for siderophorus production,while two isolates (B. cereus and B. paramycoides) showed a high capacity for ammonia production. All isolates demonstrated the ability to form biofilms, with all exhibiting high production capacity except for B. cereus, which showed a weak capacity. The isolates B.paramycoides and S. griseolus were selected to test their effect as bio fertilizers on wheat plant height, leaf area, and chlorophyll index. The results varied among the treatments.The interaction treatments between bio fertilizers and mineral fertilizers yielded the best results.The treatment with S. griseolus and a 75% level of mineral fertilization recorded the highest leaf area at 52.54 cm^2, while the interaction treatment with S. griseolus and a 25% level recorded the highest chlorophyll index at 49.43 SAPAD. S. griseolus and a 50% level recorded the highest wheat plant height at 88.30 cm.

Keywords: Algae,molecular,Biochemical and Diagnosis.

INTRODUCTION

Bio fertilizers are defined as microorganisms, or a compatible group of microorganisms, or as biological additives added to the soil to supply plants with their nutritional needs. They are sometimes called microbiaI inoculants. This technology ensures maximizing the use of beneficial microorganisms to improve the physical, chemical, and biological properties of the soil. They maintain the balance of nutrients in agricultural lands and convert them into forms available for plant nutrition (Al-Balkhi, 1990). Bio fertilizers have been used as a new method to improve plant productivity. They are considered viable,sustainable, and attractive biotechnological alternatives for increasing crop yields, improving and restoring soil fertility, and stimulating plant growth, due to theenvironmental impact associated with chemical fertilization (Sarkar et al., 2021).

Kumar (2010) pointed out the importance of using biofertilizers in providing some of the important nutrients for the plant, such as nitrogen, phosphorus and potassium, as well as secreting some hormones and acids that act as regulators of plant growth, as well as secreting some antibiotics, which helps to resist some diseases endemic in the soil and benefits the plant and its production. It is no secret that these bio-fertilizersplay a role in the environment, and their results appear directly on the plant, the soil, and the environment.They increase the plant's ability to absorb nutrients and water from the soil. The use of bio-fertilizers also reduces the large quantities of added mineral fertilizers to sometimes 50% of the recommended amount, which leads to obtaining a clean, high-quality, and safe product.They also heIp increase production, as the amount of crop produced

can reach more than 30% in some cases. They help increase the crop's content of compounds or nutrients compared to mineral fertilizers, reduce the costs of chemical fertilizers and spraying fees,and reduce the pesticides used in chemical control. In addition, the prices of bio-fertilizers are lower compared to mineral fertilizers, and the profit returns from increased production (Harman,2000).

The types of bacteria added as biofertilizers vary, as do their uses.Some are phosphorus-solubilizing, such as Achromobacter spp., Bacillus spp., Flavobacterium spp.,Brevibacterium spp., and Streptomyces spp., while others release potassium,such as Enterobacter spp., Thiobacillus thioexidans, Nitrobacter uniagradablyi, and Thiobacillus spp.(Al-Haddad, 1998). Still others fix nitrogen, such as Rhizobia and Azotobacter. To identify the most efficient biofertilizers,this experiment was conducted. It aimed to study the biochemical properties of bacteria associated with algae from the aforementioned areas,perform molecular characterization of bacteria isolated from the soil and water of Sawa Lake,and investigate certain characteristics that contribute to their ability to promote plant growth.

MATERIALS AND METHODS

Water samples with prominent algal growth were collected from natural water reservoirs (Euphrates River, Atshan River, Third River) in Iraq. The samples were placed in 50 ml Falcon tubes. One gram of algae was transferred to a 10 ml centrifuge tube and centrifuged at 1000 rpm for 5 minutes. Only the filtrate was taken. After adding the filtrate to 90 ml of sterile water in a 250 ml glass flask and mixing well, a series of dilutions were carried out by transferring 1 ml of the suspension to a test tube containing 9 ml of sterile water. The process was repeated until a dilution of-6 to 10 was reached. Then,1 ml of each dilution was taken and inoculated into test tubes containing 9 ml of nutrient broth medium,with three replicates for each dilution. The tubes were then incubated aerobically at 28°Cfor 48 hours, 0.1 ml was taken from the tubes that gave a positive indicator and spread on the surface of a Petri dish containing the solid Nutrient agar medium. The dishes were incubated at a temperature of 28°C for 48 hours. The growing bacteria were re-cultured by stripping in order to obtain pure colonies of bacteria on the Nutrient agar medium and were kept until the rest of the diagnostic tests were completed on them.

Biochemical diagnosis of bacterial isolates

Bacterial isolates were identified based on microscopic characteristics and biochemical tests as reported in (Baron and Finegold, 1990, and Collee et al., 1996), where biochemical characteristics were studied as reported in (Cruickshank et al., 1975), and included tests (Gram stain, Motility test, Voges Proskauer test, Starch hydrolysis test, Nitrate reduction,Indole test, Oxidase, Catalase, Urease test, Methyl Red, Gelatin hydrolysis test,Citrate Vtilization test,and H2S.)

Molecular diagnosis of bacterial isolates

The bacterial isolates used in the study were characterized in stages that included DNA extraction, primer preparation, DNA purity assessment, and agarose gel electrophoresis,according to the method recommended in the Taq-PCR PreMix Kit (produced by the Korean company). The same method was followed by Al. (Kraety et al. 2020). The DNA sequences were analyzed to verify their similarity to DNA sequences available in the National Center for Biotechnology Information (NCBI) database, the National Library of Medicine (NLM),and the National Institutes of Health (NIH). The DNA sequences were used to perform a BLASTN search in the sequence database (Altschul et al., 1997).

Ammonia Production

Isolates were tested for ammonia production by inoculating them in 10 mnL of sterile peptone water in test tubes. The tubes were incubated for 48-72 hours at36±2^∘ C.Nissler's reagent (0.5 mL) was then added to each tube. A change in the medium's color from brown to yellow was taken as a positive test for ammonia production (M. Nissipaul et al., 2017).

Siderophore Production

Siderophore production was qualitatively estimated. The culture media were centrifuged at 6000 rpm for 10 minutes. A 0.5% filtrate was taken and added to 0.5 mL of 0.2% aqueous ferric chloride solution. The appearance of an orange or reddish-brown color indicates the presence of Siderophore Yeole (Dube, 2000).

Hydrogen cyanide production

HCN production was detected using the Castric method (Castreck, 1983). Bacterial isolates were cultured on Nutrient agar and MacConkey agar and incubated for 24 hours. A Whatman No. 1 filter paper disc, the same diameter as a Petri dish, impregnated with picric acid solution (0.5%picric acid (w/v) in 1% sodium carbonate), was placed on the top of the Petri dish and incubated at28±2^∘ Cfor 48-72 hours under sterile conditions. No color change from yellow to light brown or strong reddish-brown indicated HCN production.

Experiment to investigate the ability of bacterial isolates to form a biofilm. Bacterial isolates were cultured intest tubes containing 10 ml of Tryptic soya broth medium,which was prepared according to the manufacturer's instructions (oxoid) by dissolving 30 g of the medium in 1 liter of distilled water, then sterilized in an autoclave at a temperature of

121°Cand a pressure of 15 p/in for 20 minutes. After incubation, the thickness of the biofilm was measured using a ruler. The contents of the tubes were poured out and 10 ml of 0.1%safranin dye was added to them and left for one minute, then the dye was poured out.The appearance of the red ring indlicates the formation of a biofilm (Christesen et al., 1982).

Experiment to Investigate the Ability of Bacterial Isolates to Form Biofilms

Bacterial isolates were cultured in test tubes containing 10 ml of Tryptic soy broth medium,prepared according to the manufacturer's instructions (oxoid) by dissolving 30 g of the medium in 1 liter of distilled water. The medium was then sterilized in an autoclave at 121°Cand a pressure of 15 psi for 20 minutes. After incubation, the thickness of the biofilm was measured using a ruler. The contents of the tubes were poured out, and 10 ml of 0.1%safranin stain was added. The mixture was left to stand for one minute, and then the stain was discarded. The appearance of a red ring indicates the formation of a biofilm (Christesen et al.,1982).

Biological Experiment

A field experiment was designed to study the effect of adding the two best isolates of bacteria, single and double, and their interaction with four levels of chemical fertilizers according to a Randomized Completely Tlock Design (RCTD) using two experimental factors and three replicates. The experiment included two factors: the first factor was biofertilization,which included four levels (no addition of B. paramycoides,S. griseolus, B.paramycoides and S. griseolus), and the second factor was four levels of mineral fertilization (0%,,25%,50%,and 75%) of the fertilizer recommendation.

After preparing the land by plowing, smoothing, and leveling, it was divided into plots measuring (1.5)x1.m2) for the experimental unit, with three sections, each section containing 16 experimental units. Planting was done in rows wvith a spacing of 20 cm between rows. Planting took place on 15-11-2024,using a seed rate of (120 kg/hectare) for the 'Ibaa 99' variety. Fertilizers were added at three levels: one-quarter, one-half, and three-quarters of the recommended fertilizer application rate for nitrogen fertilizers in the form of urea(46%N),applied in two doses: the first at planting and the second during the elongation stage.The recommended fertilizer application rate is 160 kg N ha-1. Phosphate fertilizer was added in a single application at planting in the form of triple superphosphate (20% P).Potassium fertilization was also carried out in a single application at planting in the form of potassium sulfate (50%K_2 O). The recommended fertilizer application rate is 100 kg/h-1.The crop was harvested after full maturity on 11/4/2025.

RESULTS AND DISCUSSION

Biochemical Tests

The results of the biochemical tests (Table 1) performed on bacterial isolates obtained from the water and soil of Sawa Lake showed the following:

Bacillus cereus: A Gram-positive rod-shaped bacterium whose colonies appear dark brown,irregular,large,shiny, and slimy on motile Nutrient Agar. The isolate yielded positive results for enzyme tests, including oxidase and catalase, as well as the methyl red test and nitrate reduction. It also showed negative results for the Fox-Proscour assay, the indoleassay,and the gelatinase assay. The isolate exhibited the ability to produce urease, but it was unable to hydrolyze starch.

Bacillus paramycoides: A Gram-positive, rod-shaped bacterium. Colonies on nutrient agar are cream-colored, filamentous, and hair-like. The isolate showed immobility. It also showed positive results for catalase and methyl red enzyme tests, and produced urease, in addition to being able to produce indole. The isolate also showed the ability to reduce nitrates, but was unable to produce oxidase and consume citrates, and was unable to hydrolyze starch. It also recorded negative results for fuchs-Proscour, gelatinase, and citrate tests.

Streptomyces griseolus:A Gram-positive,filamentous bacterium. Colonies appear on Nutrient Agar as ice-gray, toothed, immobile colonies. The isolate yielded positive results for enzyme tests (oxidase, catalase, and erythrocyte sedimentation rate). It also showed a positive result for the urease test and demonstrated the ability to hydrolyze starch and reduce nitrates. However, the isolate showed negative results for the Fox-Proscor test, citrate consumption, indole assay, and gelatinase assay.

Klebsiella aerogenes: A Gram-negative,rod-shaped, white, convex,shiny,mucous-like,large, regularly shaped, motile bacterium. The isolate yielded positive results for catalase,urease,methyl red, indole, and nitrate reduction enzyme tests,while it showed negative results for oxidase, starch breakdown, fuchs-proscautery,citrate consumption, and gelatinase enzyme tests.

Citrobacter freundii: Its colonies are large,round, convex, semitransparent, smooth, mucous-like, Gram-negative,motile rod-shaped bacteria. The isolate also showed positive results for catalase and nitrate reduction enzyme tests, as well as the ability to consume citrate and break down starch. However, it showed negative results for methyl red and fuchs-proscautery tests. It also exhibited an inability to produce oxidase and indole enzymes.

Isolation number / Tests	Bacillus cereus	Bacillus paramycoides	Streptomyces griseolus	Klebsiella aerogenes	Citrobacter freundii
Gram stain	+	+	+	-	-
Motility test	+	-	-	+	+
Voges proskauer test	-	-	+	-	-
Starch hydrolysis test	-	-	+	-	┼
Nitrate reduction!	+	┼	+	+	+
Indol test	-	+	-	┼	-
Catalase	+	-	+	-	-
Oxıdase	+	+	+	+	+
Urease test	+	+	+	+	+
Methyl Red	+	-	+	+	+
Gelatin hydrolysis test	-	-	+	-	-
Citrate Vtilization test	-	-	-	-	+

Table (1) Results of biochemical tests for algal-associated bacterial isolates

The results shown in Table (2) demonstrate the ability of all bacterial isolates to produce ammonia,based on the development of a yellow color. These isolates were classified as weak,moderate, and strong. The table also reveals variations in ammonia production among the isolates,with B. cereus and B. paramycoides outperforming the others. All isolates exhibited high efficiency in siderophore production, as evidenced by the reddish-brown color change of the medium after detection. The results also indicate the high production capacity of isolates B. paramycoides, S. griseolus, K. aerogenes, and C. freundi. However, the results of the HCN production test indicate moderate capacity for all four isolates used in the experiment, except for K. aerogenes,which showed weak HCN production.

Table (2) The ability of isolates to produce (HCN, Ammonia, Sideophore and biofilm)

isolation	HCN	Ammonia	Siderophore	biofilm
1	nability	to produee//+$weaK$ ‖ +Hf + $Average$	+	
2	++	xx	+++	+++
3	++	++	+++	+++
4	+	++	+++	+++
5	++	++	+++	+++

Effect of Biofertilizer and Mineral Fertilizer and Their Interaction on Leaf Area (㎠)

Statistical analysis results indicated a significant effect of bio fertilizer and mineral fertilization treatments and their interaction on leaf area concentration. Table (3) showed the superiority of the interaction between the two isolates, as the highest average leaf area was recorded at 45.85 ㎠. This increase may be attributed to the role of bio fertilizers in increasing the availability of nutrients, including nitrogen, which plays an imnportant role in the formation of protoplasm, cells, and cell membranes. Nitrogen is involved in the formation of enzymes and coenzymes, namely NADH and NADPH, and vitamins,specifically the B complex. It is also involved in some growth regulators and is a component of chlorophyll, making it essential for photosynthesis and a component of nucleic acids (Nuqta & Al-Shater,2011).

The table results indicate that the 75% level of mineral fertilization was superior to the other levels, recording an average of 49.66 ㎠. This aligns with (Al-Haidari's 2003) findings,which noted an increase in flag leaf area in wheat

with increasing nitrogen levels. This may be attributed to nitrogen's role in meristematic cell activity and leaf area enhancement, as well as its significant contribution to auxin production, which stimulates vegetative growth.The table also shows that the interaction coefficients between bio fertilization and chemical fertilizers were superior for the single S. griseolus isolate and the combined B. paramycoides +S.griseolus isolate, with the 75% level of mineral fertilization recording averages of 52.54c㎡ and 50.01 c㎡,respectively.

Table (3) Effect of bio-fertilization and chemical fertilization treatments and their interaction on the leaf area (cm2) characteristic of wheat

Treatment Isolation	Chemical fertilizer levels kg.h-1 %0	%25	%50	%75	45.2
TO	44.46	49.16	44.27	42.91	43.72
B.paramycoides	42.74	40.07	40.90	51.19	45.4
S.griseolus	41.86	47.07	40.13	52.54	45.80
S.+B.paramycoides griseolus	40.40	46.20	44.50	52.01	
average	42.37	45.63	42.45	49.66	
0.05L.S.D	$T \times C = 13.07$	$C = 6.53$	$T = 6.53$		

Effect of Bio-fertilizers, Mineral Fertilizers, and Their Interaction on Height(cm)

Statistical analysis results indicated a significant effect of bio-fertilizers,chemical fertilizers,and their interaction on wheat plant height. Table (5) showed the superiority of the S.griseolus isolate, which recorded the highest average height of 82.14 cm. This may be attributed to the important role of the bacterial isolate in metabolic processes, increasing the rate of cell division, elongating stem internodes, producing growth promoters,and its ability to supply nutrients to plants. These results are consistent with those of (Schoebitz et al.,2013). The results also showed a significant increase in plant height when the level of chemical fertilization increased, as the fertilization level of 75% exceeded all levels with an average of 83.49 cm, while the control treatment recorded the lowest average of 74.42 cm.This may be due to the increase in the amount of added macronutrients NPK to the soil,which increased its concentration and consequently increased the amount absorbed by the plant, which was reflected positively on plant growth and increased its height as a result of better root system growth and its reflection on increasing the concentration of phosphorus in the soil. These results are consistent with what was concluded by (Al-Rawi et al., 2001).

On the other hand, the results indicated significant differences in the interaction between bio fertilizer and mineral fertilizer in the plant height characteristic. The highest value for plant height was in the interaction(50%×S.griseolus), which reached 88.3 cm. This may be attributed to the support of bio fertilizers with half the fertilizer recommendation, as the mineral fertilizer works to provide the necessary nutrients for the growth of organisms,which in turn leads to an increase in the growth and development of the biofilm by increasing the size of the colonies, which is reflected positively on the biological activity of the organisms that make up the bio fertilizer (Seneviratne et al., 2011).

Table (5) Effect of bio-fertilization and chemical fertilization treatments and their interaction on the height characteristic of wheat plants (cm)

Treatment Isolation	Chemical fertilizer levels kg.h-1 %0	%25	%50	%75	
TO	73.26	80.80	81.13	78.60	78.45
B.paramycoides	78.77	78.77	72.97	83.30	78.45
S.griseolus	74.6	79.57	88.30	86.07	82.14
B.paramycoides S.griseolus +	71.03	79.03	84.63	86.00	80.17
average	74.42	79.54	81.76	83.49	
0.05L.S.D	$T \times C = 11.372.$	$C = 5.686$	$T = 5.686$		

CONCLUSION

In this study, five Cladophora algal isolates were identified molecularly. These isolates demonstrated the ability to produce biofilms, ammonia, siderophore, and HCN. Two isolates,B.paramycoides

and S. griseolus, were selected for use as biofertilizers in the biological experiment. S. griseolus exhibited superior characteristics in plant height, leaf area, and chlorophyll index. We recommend further experiments to determine the potential of these isolates for biofertilization and biological control.

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