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Characterizations and Identification of Promoting Plant Growth and Bioagents Bacterial Strains as Indicators for its Productions

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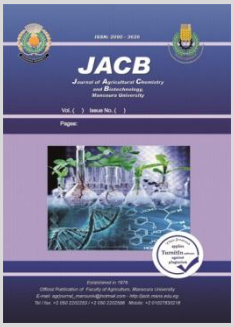

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ABSTRACT

Drought-tolerant bacterial strains used for promoting plant growth include groups of bacteria that can directly enhance plant development and indirectly control fungal phytopathogens. In this study, bacteria were isolated from the nodules and rhizosphere of soybean plants. Using classical microbiological methods, two isolates were identified based on cultural, morphological, biochemical, and physiological characteristics *in vitro*. The first strain, designated as DTB4, was isolated from the nodules of soybean plants. It was classified as a slow grower, aerobic, gram-negative, short rod-shaped, non-spore-forming, and motile. Optimal growth occurred at 35°C and pH 7.0. It tested positive for starch, casein, and gelatin hydrolysis, as well as for the indole test. The strain was also capable of assimilating several sugars and showed resistance to certain antibiotics. The second strain, designated as DTR30, was isolated from the rhizosphere of soybean plants. It consisted of long rod-shaped, gram-positive, spore-forming, oxidase-negative bacteria. Biochemical tests revealed positive results for indole and methyl red. This strain tolerated NaCl and exhibited growth at pH 9.5. Based on identification tests, strain DTB4 was confirmed to be *Bradyrhizobium japonicum*, and strain DTR30 was identified as *Bacillus subtilis*. Biochemical analysis indicated that both strains have the potential to produce several products for using in different scales.

Keywords: Bacillus, Bradyrhizobium, Identification, Standard tests

INTRODUCTION

Bradyrhizobium japonicum is the most common microsymbionts of soybean (Zhang *et al*. 2011). Genus of Bradyrhizobium contain seven species this genus of Bradyrhizobium is short rod shaped (0.5-0.9 µm X 1-2x3.0 µm), gram negative, motile by polar or subpolar flagellum, non-spore forming. Strains of *B. japonicum* are the slow growing and aerobic with oxygen as the terminal electron acceptor when possessing a respiratory type of metabolism (Salvagioth *et al*. 2018).

Rhizobia are known for supplying plants with nitrogen and as an excellent biological control agent against soil-borne pathogens (Deshwal *et al.* 2003). Nitrogen fixation by rhizobia can improve plant growth and this microorganism can availability nutrients and minerals. Similarty, rhizobia are able to produce secondary metabolites such as alkaloids and flavonoids that improve plant defense against pathogens (Hasan *et al.* 2020 & Basu *et al.* 2021). Biological nitrogen fixation, plant growth and thereby crop yield potential by symbiotic between *Rhizobium*-legumes, are factors effects on adverse environmental conditions (drought stress, salt stress, acidity/alkalinity, nutrients deficiency, heavy metals and various pesticides used to decrease diseases (Ghalgir *et al.* 2021). The bacteria *Bradyrhizobium japonicum* the best use in production of biofertilizers (Ghazala *et al.* 2023). By rotated with crop in farming system, legumes and Rhizobium can provides value to control weed, pathogens and insect (Afify and Ashour 2024).

A large group of microorganisms are *Bacillus subtilis*, it is a common aerobic soil bacterium. One from this group *Bacillus* species are widely distributed in the environment and are Gram-positive, spore-forming bacteria. Members of genus Bacillus such as *B. subtilis*, *B. amyloliquefaciens* and *B. velezensis* have been proven to have a great influence on plant growth and antagonistic effects on various pathogens in several plants. One of the important characteristics of *Bacillus* spp. is that they produce some antibiotics. In recent years, these antibiotics are considered antifungal activity low toxicity and allergenicity. In addition, resistance to antibiotics are very important factor used to characterize between different bacterial strains by reported resistance to antibiotics for colony morphology (Sinclair and Eaglesham 1984). Nowadays, rapid identification of bacterial isolates will be made easier with the use of standard biochemical identification kits (Hildebrand *et al.* 1992). To the protective effect, many strains of microorganisms also have properties that stimulate plant growth these isolates were belongs to the genus Bacillus by biochemical tests (Shraddha *et al.* 2022). In the present investigation the best bacterial isolates as biofertilizers and as bioagents for plant diseases were obtained to identical of their cultural, morphological, biochemical and physiological characterizations.

MATERIALS AND METHODS

Source of bacterial isolates

The present section describes the bacterial isolates, were obtained as drought-tolerant bacteria that enhance the growth of plant directly and /or biocontrol agents indirectly. The double (DTB 4 & DTR 30) active bacterial isolates were selected for identification via standerd tests are presented in Tables (1 &2). Important notes: DTB= drought-tolerant bacteria and DTR= drought-tolerant rhizosphere

Molecular characterization bacterial strains DTB 4 and DTR 30 using PCR-Ribotyping

Molecular characterization using PCR was carried out by sequencing the 16S rRNA gene of both strains by Nader *et*

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al. (2024). From molecular results were identical that the isolates DTB 4 belonged to *Bradyrhizobium japonicum* PP236808 and isolate DTR30 belonged to *Bacillus subtilis* PP250150also with compatibility of 100% and with probability of 95 % when compared with biochemical tests (Dhuha and Hameed 2020). .

Table 1. The activities of the most efficient drought-tolerant bacterial strains directly (Nader *et al*. 2024)

Bacterial strains	IAA (mg/100 ml)	GA (mg/100 ml)	Proline (mg/100 ml)	EPS (mg/ L)	Phosphate solubilization (mg/100ml)
DTB 4	18.66±0.13a	194.46±0.41c	55.07±0.29c	86.23±0.22a	47.4±1.11a
DTR 30	14.44±0.07a	188.09±8.04b	74.5±0.13a	60.35±0.06a	68.92±0.37e

IAA, Indole acetic acid; GA, Gibberellic acid; EPS, Exopolysaccharides. Data are means ± SD (n=3); different letters within the same group indicate significant differences between means according to Duncan's multiple-range test at $P \leq 0.05$.

Table 2. Inhibition mycelial growth of two soil-borne fungi by drought-tolerant bacterial strains indirectly (Afify and Ashour 2024)

Treatment	Inhibition zone (mm)		Means (mm)
	F. oxysporum	*R. solani*	
DTB 4	11	11.5	11.25
DTR 30	13	12	12.5
Control (only fungus)	0.0	0.0	0.0

0.0 =full growth of fungus = no inhibition zone

Standard tests for identification bacterial strains DTB 4 and DTR 30

These tests as cultural, morphological, biochemical and physiological characteristics of two bacterial strains DTB 4 and DTR 30 were carried out using standard methods described in Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons 1974) were studied.

1.Morpho-cultural characters of drought-tolerant bacterial strains

Morphological and microscopic characteristics of both the strains were investigated. For both strains (DTB 4 and DTR 30) after an incubation of 3 to 5 days at 28 ± 2ºC (yeast extract mannitol agar plate for growth DTB 4 and nutrient agar for growth DTR 30). Individual colonies were characterized based on shape, size, color, margin etc. were observed at 72-96 hrs. of incubation. By microscopically cell shape, cell size, motility, gram, spore stains and catalase were recorded (Pawar *et al.* 2014).

2.Biochemical characteristics

Biochemical test like starch, casein, lipids, gelatin hydrolysis and tests such as indole, methyl red, voges-proskauer, citrate utilization, H_2S production and nitrate reduction were also done according to classical identification methods.

3. Physiological characteristics

Effect of NaCl concentrations

Yeast extract mannitol broth and nutrient broth were inoculated with loopful cultures individually of both strains (DTB 4 & DTR 30) respectively and were incubated at different concentrations from 1%, 2% and 6.5% NaCl to find out the effect to salt on strains growth. By using spectrophotometer, growth of bacterial strains were measured as turbidity at 600 nm.

Effect of pH levels

Effect of pH levels on growth of both strains was studied by adjusting pH of the YEMA and NA media to various levels *viz*., 4.5, 7.0, 9.0 and 9.5 using appropriate phosphate buffer. After the incubation period observations were recorded for the growth of bacterium by using spectrophotometer at 600 nm.

Effect of temperature

Yeast extract mannitol broth and nutrient broth were inoculated with loopful cultures individually of both strains (DTB 4 & DTR 30) respectively and were incubated at different temperature ranging from 10ºC to 45ºC (10, 15, 20, 30, 35, 40 and 45ºC) to find out the effect to temperature on strains growth and measured of growth turbidometrically.

Evaluation of antibiotic sensitivity

Testing the resistance or sensitivity of bacterial strains against different antibiotics by antibiotic disc assay according to Ahmad *et al.* (2001). The following antibiotics from Sigma Company Egypt, purchased included Penicillin, Kanamycin, Novobiocin, Streptomycin and Tetracycline.

RESULTS AND DISCUSSION

To study the cultural properties, the bacterium isolate DTB 4 grew better on YEMA plates. The colonies developed were circular, convex, glistening, whitish pink, with entire margin and measured about 2-4 mm. On the case of bacterium DTR 30 culture was grown on 2% meat peptone agar (MPA) for 1-3 days. Next, the features of the colonies were studied in Petri dishes, and the morphology of the cultures under a microscope. Milky-white velvety colonies with uneven wavy edges and a viscous consistency were observed on the surface of the MPA (Table 3).

Table 3. Cultural characteristics of bacterial strains DTB 4 and DTR 30

Characters of culture	Bacterial strains	
	DTB 4	DTR 30
Media for better growth	YEMA	MPA
Shape	Circular	irregular
Raise	Slightly - Convex	curly
Velvet	Glistening	Viscous
Color	Whitish pink	Milky-white
Margin	Entire	uneven wavy
Size (mm)	2-4 (Pinpoint,Small)	5-7

YEMA =Yeast Extract Mannitol Agar
MPA= Meat Peptone Agar

Drought-tolerant bacterial strain DTB 4 cells were rod-short shaped, gram-negative, non-spore-forming, motile, non-pigments formed in old cultures, and catalase-positive. Hydrolyzing starch, not hydrolyzing casein and lipids, positive for indole and negative for V. P. tests, can assimilate glucose, fructose, mannitol, sucrose, and starch as sole carbon sources. In addition, drought-tolerant bacterial strain DTR 30 cells were rod-long shaped with a diameter of 0.5– 0.7 µm and a length of 3.5–5.0 µm, gram-positive, spore-forming, motile, non-pigmented formed in old cultures, and catalase-positive. Hydrolyzing starch, casein, and lipids, positive for Indole and V. P. tests, can assimilate glucose, fructose, mannitol, sucrose, citrate and do not ferment lactose, maltose, rhamnose and galactose as sole carbon sources. The morphological and biochemical properties of bacterial strains DTB 4 and DTR 30 are presented in Tables (4 & 5).

To study the physiological properties, the bacterium isolate DTB 4 grew and tolerant bacterial strain for acidity (pH 4.5 and 7.0), for NaCl (1.0 and 2.0%) and optimum

growth was 35ºC. While, the bacterium isolate DTR 30 grew best at 30ºC and 35ºC and tolerant to 40ºC and 45ºC. Range growth for NaCl were 1% to 2% and tolerant for acidity to pH 9.5. The physiological characteristics of bacterial strains DTB 4 and DTR 30 are presented in Table (6).

Table 4. Morphological characteristics of bacterial strains DTB 4 and DTR 30

Characters of cells	Bacterial strains	
	DTB 4	DTR 30
Cell shape	rod-short	rod-long
Diameter (µm)	0.4-0.25 x 1.25-3.00	0.5– 0.7 x 3.5–5.0
Motility	+	+
Gram reaction	-	+
Sporulation	-	+
Catalase test	+	+

Table 5. Biochemical characteristics of bacterial strains DTB 4 and DTR 30

Chemical test	Bacterial strains	
	DTB 4	DTR 30
Hydrolysis of:		
Starch	+	+
Casein	+	+
Lipids	-	+
Gelatin	+	+
Indole test	+	+
Methyl Red (M.R.) test	-	+
V.P. (Voges-Proskauer) test	-	-
H_2S production	-	-
Nitrate reduction	-	-
Assimilation of sugars:		
Glucose	+	+
Fructose	+	+
Mannitol	+	-
Sucrose	+	+
Citrate	+	+
Lactose	+	-
Maltose	+	+
Rhamnose	-	-
Galactose	+	-
Antibiotic resistance:		
Penicillin	-	-
Kanamycin	-	+
Novobiocin	-	+
Streptomycin	-	-
Tetracycline	-	-

+ = positive reaction ; – = negative reaction

Table 6. Physiological characteristics of bacterial strains DTB 4 and DTR 30

Growth at:	Bacterial strains	
	DTB 4	DTR 30
1 % NaCl	+	+
2 % NaCl	+	+
6.5 % NaCl	-	+
pH 4.5	+	
pH 7.0	+	+
pH9.0	-	+
pH9.5	-	+
10ºC	-	-
15ºC	-	-
20ºC	-	+
30ºC	++	++
35ºC	+	++
40ºC	-	+
45ºC	-	+

= no growth ; + = normal growth ; ++ = highly growth

From data in the present investigation bacterial isolates identified were applied on the standard tests according to identical tests it was identified that isolate DTB 4 is *Bradyrhizobium japonicum* and isolate DTR30 is *Bacillus subtilis*.

Results of the bacterial characterizations revealed that most of the isolates were catalase and urease, nitrate reduction positive and unable to utilize citrate, which complements with the results of Lupwayi and Hague (1994). The antibiotic resistance were used for repoted variants of isolates by colony morphology (Sato 1995). The most important biochemical tests *viz*. catalase oxidation, potassium hydroxide (KOH) solubility and starch hydrolysis of *B. japonicum* were attempted (Aneja, (2003). All the isolates showed negative results for starch hydrolysis. It was also observed that the isolates did not produce gelatinase enzymes. Negative gelatinase activity of *Rhizobium* was also observed by Hunter *et al* (2007). The results also confirmed that the biochemical features of all the isolates except DCJ3 and HCJ3 are similar to the biochemical features of reference strains, *Rhizobium leguminoserum* MTCC-99 and *Mesorhizobium thiogangeticum* MTCC-7001. Similar to the present study Deshwal *et al* (2014). Also, results confirm that with the Bacillus isolates (Enez 2020). Biochemical test like starch hydrolysis, casein hydrolysis, gelatin hydrolysis, indole test, methyl red test, voges-proskauer test, citrate utilization test, H_2S production test, nitrate reduction test and catalase test were also done (Shoaib *et al.* 2020). With the key morphological and biochemical traits spore forming bacteria were similar to such of reference strain *B. subtilis* were identified to *Bacillus subtilis* (Honchar *et al.* 2021). When bacterial isolates were obtained from nodules and rhizosphere of soybean plant (Anandrao *et al.* 2022 & Ghazala *et al*. 2023).

CONCLUSION

Two drought-tolerant bacterial strains were isolated from soybean plants and identified using cultural, morphological, biochemical, and physiological characterizations methods. The results revealed a close genetic relationship to *Bradyrhizobium japonicum* (PP236808) and *Bacillus subtilis* (PP250150), respectively. Both bacterial strains showed promising potential as biofertilizers to promote plant growth and as biocontrol agents against fungal pathogens. Interestingly, the identification conducted through classical methods was consistent with molecular characterization results. Standard parameters such as cultural traits, morphology, biochemical reactions, and physiological characteristics closely matched molecular identification. The two strains were identified as follows: strain DTB4, isolated from soybean nodules, was confirmed to be *Bradyrhizobium japonicum*; and strain DTR30, isolated from the soybean rhizosphere, was confirmed as *Bacillus subtilis*. These strains may serve as effective biofertilizers and biocontrol agents to enhance plant growth and manage fungal diseases. Additionally, both strains demonstrated strong potential as sources of valuable products.

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خصائص وتعريف السلالات البكتيرية المشجعة لنمو النبات وكذلك كعوامل مقاومة حيوية كدلائل لمنتجاتها

عا يده حافظ عفيفى

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الملخص

تعتبر السلالات البكتيرية المتحملة للجفاف مجموعة من الريزوبكتيريا المشجعة لنمو النبات حيث أنها تعمل على تحسين نمو النبات بطريقة مباشرة أو بطريقة غير مباشرة لأنها مجموعة من البكتيريا قادرة على مقاومة فطريات التربة الممرضة للنبات. من هنا وفى هذه الدراسة تم عزل بكتيريا من العقد الجذرية ومن ريزوسفير نبات فول الصويا وباستخدام الطرق الميكروبيولوجية الكلاسيكية فى تعريف هذه البكتيريا معمليا (المزرعية, المورفولوجية, البيوكيميائية والفسيولوجية). فقد أختبرت السلالة الأولى المعزولة من العقد الجذرية لنبات فول الصويا وذلك بنموها على بيئة أجار المانيتول ومستخلص الخميرة المضاف لها أحمر الكونغو وقد سجلت النتائج هنا أنها بطيئة النمو أما باضافة البروموثيمول بلو إلى نفس البيئه فقد سجلت النتائج أنها عصويات قصيرة متحركة غير متجرثمة هوائية وسالبة لجرام وأن درجة الحرارة المثلى لنموها ٣٥ °م أما درجة الحموضة فهى ٧, أيضا سجلت هذه السلالة أنها موجبة للأوكسيديز والأميليز والكتاليز ومختزلة للنترات. أما بالنسبة للسلالة البكتيرية الثانية والمعزولة من ريزوسفير نبات فول الصويا فقد أظهرت النتائج أنها عصويات طويلة موجبة لجرام متجرثمة وموجبة للكتاليز تتحمل الملوحة وتقاوم الحموضه ٢,٥ و ٤,٠ من الدراسة الحالية وطبقا لقواعد الإختبارات القياسية المتبعة لتعريف السلالات البكتيرية فإن الأسماء العلمية للسلالتين على الترتيب: الأولى برادى ريزوبيوم جابونيكم والثانية باسيلس سنلس. كما أظهرت نتائج الإختبارات البيوكيميائية أن سلالتان البكتيريا موضع الدراسة تمتلك القدرة على إنتاج مواد متنوعة يمكن إستخدامها فى المجالات المختلفة.