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PHENOTYPIC AND GENETIC DIVERSITY OF NATIVE *RHIZOBIUM* ISOLATED FROM ROOT NODULES OF LEGUMINOUS PLANTS GROWN IN RECLAIMED SOIL, EGYPT

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SUMMARY

An investigation of the phenotypic characteristics of 19 rhizobial strains, isolated from root nodules of different plant legumes grown in the soil of agriculture farms in Ismailia governorate, transpired. Most isolates were creamy or white opaque, mucoid, with a convex elevation, translucent, and smooth margined. Microscopic investigation revealed that all bacterial isolates were rod-shaped and had no positive affinity for Gram-stain. Identifying rhizobial cultures from any bacterial contaminants employed confirmatory tests based on prepared special media, including YMA supplement with Congo red, glucose peptone agar, Kit-lactose agar, and Hoffer's alkaline test. Based on an infectivity test, all isolates proved their ability to reinfect their host. These rhizobial isolates, classified into two categories, included fast and slow-growing rhizobia according to their growth in the YEM medium containing bromothymol blue (BTB). Meanwhile, the assessment of the genetic diversity among these isolates proceeded using ISSR and RAPD markers, which ISSR marker proved a more powerful tool in discriminating among the tested isolates than the RAPD marker. The cluster analysis, with the RAPD marker, classified the isolates into two main groups. The first group included the isolates (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 13), while the second group contained the isolates (12, 14, 15, 16, 17, 18, and 19). Moreover, using ISSR markers also showed a cluster of two main groups with diverse categories; the first cluster included isolates 1 to 11, and the second group contained isolates 12 to 19.

Keywords: *Rhizobia*, ISSR, RAPD, and phenotypic characterization

Key findings: ISSR markers proved a more powerful tool in discriminating among the tested rhizobial isolates than RAPD markers.

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INTRODUCTION

In an era of rising ecological degradation, climate change, soil erosion, biodiversity loss, and global population expansion arose as major threats to food security. The only fundamental strategy and solution for such circumstances are taking control and using natural resources. Among these global problems is the use of chemical fertilizers for agriculture. The fertilizer industry delivers significant amounts of fixed nitrogen but poses a health risk to humans due to underground water and soil contamination. Applying the

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beneficial naturally occurring processes, such as those supplied by soil and plant-associated microorganisms, provides a possible technique for reducing agrochemical dependency. These associated processes by living microorganisms are known as biofertilizers, phyto-biostimulators (Elavarasi *et al*., 2020; Abdel-Lateif and Abd El-Ghany, 2023), and biopesticides (Ruiu, 2018; Kumar *et al*., 2021). Defined as living microbes, they improve plant growth and restore soil fertility through fixation, mobilizing, and increasing nutrient availability in soils. Among these biofertilizers is biological nitrogen fixation. Biological nitrogen fixation (BNF), widely used in agriculture, has captivated scientists interested in plant mineral nutrition for a century.

The first discovery of a specialized group of prokaryotes able to perform nitrogen fixation was by Beijerinck in 1901. These organisms process a particular enzyme named nitrogenase that catalyzes the conversion of atmospheric nitrogen (N_2) to ammonia (NH_3). These prokaryotes include cyanobacteria, free-living soil bacteria like Azotobacter (Wagner, 2011); filamentous actinobacteria, symbiotic relationships with non-leguminous trees (Torrey, 1978); bacteria that create associative relationships with plants like *Azospirillum*; and, a most critically important bacteria that form symbiotic relationships with leguminous plants that include *Rhizobium* and *Bradyrhizobium*.

Nitrogen-fixing rhizobia are an essential group of Gram-negative bacteria that can fix a huge amount of atmospheric nitrogen as a result of symbiosis with leguminous plants (Hakim *et al*., 2020; Khalid *et al*., 2020; Al-Kurtany *et al*., 2023). A report stated that many legume plants could fix up to 300 kg of nitrogen N ha^{-1} through their rhizobial symbiosis (Peoples *et al*., 1995). The legume symbiosis saves about 90 million t of total biological nitrogen needed for agriculture.

Identifying rhizobia is imperative and a prerequisite before applying promising candidates in the field for nitrogen fixation. The growing interest in using rhizobia as biofertilizers in agricultural systems led to identifying numerous rhizobia strains and exploring their diversity. Meanwhile, inoculants containing a variety of rhizobial strains have been recommended to promote soil fertility and symbiotic nitrogen fixation in legumes (Daiwshala *et al*., 2021). Therefore, more research needs action to find a broader range of rhizobia strains that enhance indirect plant growth by producing phytohormones and mineral uptake.

Developing several phenotypic methods progresses to identify and characterize rhizobial strains and their diversity. However, with available genome sequencing, wide use of PCR-based methodologies with DNA sequencing continues for the genetic diversity analysis of bacteria, particularly for groups that are difficult to distinguish using conventional approaches. Therefore, the molecular criteria are more efficient in assessing genetic relationships among populations of rhizobia (Rai *et al.,* 2012; Fetyan and Mansour, 2012). DNA markers, such as ISSR and RAPD, proved as excellent sources of diversity and establishing new genetic relationships (Randhawa *et al.*, 2013; Etminan *et al*., 2016; Fathy *et al*., 2021). RAPD use is one of the quickest and easiest markers to discriminate *Rhizobia* on strains level (Rajasundari *et al*., 2009; Shoukry *et al*., 2013; Bhuyan *et al.,* 2014; Flores-Félix *et al*., 2020). Meanwhile, ISSR is another good marker for evaluating genetic diversity, characterized by high polymorphism and recurrence (Zhang *et al*., 2016). The study goal was to use morphological and biochemical assays to characterize distinct rhizobial isolates retrieved from various nodulated leguminous plants, along with ISSR and RAPD markers, to assess genetic diversity among them.

MATERIALS AND METHODS

Sample collection and isolation technique

Root nodules from various leguminous plants grown in different localities were collected (Table 1). Investigating the soil surrounding the root system evaluated the soil type and the available essential nutrients. For isolation, cleaning the nodules and slicing small pieces preceded insertion into Petri dishes with Yeast Extract Mannitol Agar (YEMA) media supplemented with Congo red. Incubating the Petri dishes at 28 °C ± 2 °C for 48 h followed. Taking the subsequent outgrowth and spreading once more on the same medium obtained pure colonies. An obtained single colony propagation continued on YM broth.

Table 1. The designation code of the *Rhizobium* isolates recovered from root nodules of different leguminous plants.

Isolate code	Host origin		Family	Location
	Scientific name	Local name (English/Arabic)		
1	*Melilotus indicus* (L.) All.	Yellow sweet clover	Fabaceae	ReviTec, Suez Canal Univ., Ismailia
2	*Trigonella foenum-graecum*[*]	fenugreek/ Helba	Fabaceae	Sarabiyum, Ismailia
3	*Trigonella foenum-graecum*	fenugreek/ Helba	Fabaceae	Botanical Garden, Ismailia
4	*Trigonella foenum-graecum*	fenugreek/ Helba	Apiaceae	ReviTec, Suez Canal Univ., Ismailia
5	*Vicia faba* L.	Broad bean/Fouel	Fabaceae	Botanical Garden, Ismailia
6	*Arachis hypogaea L.*	Groundnuts/Fouel Sowdany	Fabaceae	ReviTec, Suez Canal Univ., Ismailia
7	*Acacia saligna*[*]	Orange wattle/Elsant tree	Fabaceae	ReviTec, Suez Canal Univ., Ismailia
8	*Glycine max* (L.) Merr.[*]	Soybean/ Fouel soya	Fabaceae	Botanical Garden, Ismailia
9	*Trifolium alexandrinum*	Berseem clover	Fabaceae	Tell El Kebir, Ismailia
10	*Glycine max* (L.) Merr.[*]	Soybean/ Fouel soya	Fabaceae	Botanical Garden, Ismailia
11	*Phaseolus vulgaris*	Navy bean/ salad bean/ fasolia	Fabaceae	Tell El Kebir, Ismailia
12	*Lupinus termis – Forssk*[*]	White Lupin/Termis	Fabaceae	Agriculture Farm, Suez Canal, Ismailia
13	*Vicia faba* L.	Broad bean/Fouel	Fabaceae	Veterinary Farm, Suez Canal Univ., Ismailia
14	*Acacia Saligna*[*]	Orange wattle/Elsant tree	Fabaceae	ReviTec, Suez Canal Univ., Ismailia
15	*Cicer arietinum*	Chickpea/Homos	Fabaceae	Agriculture Farm, Suez Canal, Ismailia
16	*Vicia faba* L.	Broad bean/Fouel	Fabaceae	Sarabiyum, Ismailia
17	*Phaseolus vulgaris*	Navy bean/salad bean/ fasolia	Fabaceae	Botanical Garden, Ismailia
18	*Lupinus termis – Forssk*[*]	Chickpea/Homos	Fabaceae	Agriculture Farm, Suez Canal, Ismailia
19	*Trigonella foenum-graecum*[*]	fenugreek/ Helba	Fabaceae	Sarabiyum, Ismailia

[*] Nodules from plants marked with an asterisk were subjected for isolation twice for two consecutive years.

Morphological characterization

Morphological characteristics of the propagated colonies ensued. Individual colonies' characterization consisted of size, color, shape, and elevation following two to five days of incubation at 28 °C on the YEMA plate. On the other hand, micromorphology examination used a microscope with Gram stain. For further studies, the most prominent isolates remained on YEMA slants in a refrigerator at 4 °C.

Growth on YMA containing bromothymol blue

The isolated strains were streaked on YEMA medium supplemented with bromothymol blue and incubated at 28°C ± 2°C for 3-4 days. The pH of the medium was recorded to determine whether the selected pure isolates produced any acids or alkalis, and to monitor the growth and changes in the medium over time.

Confirmatory tests

A special media preparation was used, which included a YMA supplement with Congo red, glucose peptone agar, Kit-lactose agar, and Hoffer's alkaline test to identify rhizobial cultures from any bacterial contaminants (Nelson and Child 1981). All pure isolates were examined on this media to determine if they belonged to Rhizobium cultures and if they were free of *Agrobacterium* contamination. The isolates were streaked onto plates for each medium and incubated for 48 hours at a temperature of 30°C ± 2°C. The colony appearance and pH changes were then documented.

Biochemical tests

All the pure bacterial isolates recovered from the root nodules of different leguminous plants underwent biochemical characterization including tests for catalase, urease, nitrate reduction, starch hydrolysis, gelatin liquefaction, citrate utilization, and hydrogen sulfide production. The ability to ferment some sugars, such as, D-glucose, mannitol, and L-arabinose, were also done

Infectivity test

Each plant host's seeds were surface sterilized, according to Mansour *et al.* (2003). Placing

sterilized seeds on sterile, wet filter paper allowed them to germinate in Petri plates for two days at room temperature. After that, moving the seedlings aseptically to growth pouches made of transparent plastic followed. All acquired isolates gained inoculation into seedlings, each seedling receiving 0.01 packed cell volume of each propagated isolates. Then, placing the inoculated seedling in a growth chamber sought to confirm the ability to produce nodules.

DNA isolation technique

Pure rhizobial strains were grown for 24 to 48 h before undergoing DNA extraction. The bacterial DNA extraction used the QIAprep Spin Miniprep Kit (QIAGEN). The QIAprep Miniprep technique relies on the alkaline lysis of bacterial cells, followed by the adsorption of DNA onto silica in the presence of high salt concentrations.

RAPD analysis

PCR, according to the method of Williams *et al.* (1990), used six RAPD primers (Table 2). The DNA amplifications were as follows: in an automated thermal cycle (model Techno 512) programmed for one cycle at 94 °C for 4 min, followed by amplification 30 cycles of 94 °C for 45 s, 37 °C for 45 s, and 65 °C for 2 min 30 s. Finally, the stored reaction was at 72 °C for 10 min. Photograph of PCR products was under UV light.

ISSR analysis

PCR reactions followed the method according to Hussein *et al*. (2006) using six ISSR primers (Table 3)**.** The DNA amplifications proceeded in an automated thermal cycle (model Techno 512) programmed for one cycle at 94 °C for 4 min, followed by 45 cycles of 1 min at 94 °C, 1 min at 57 °C, and 2 min at 72 °C. The final reactions got stored at 72 °C for 10 min. PCR products analysis using gel electrophoresis (1.5% agarose gel), then photographed under UV light.

Data analysis

A FORTRAN software for organizing multivariate data (Hill, 1979) in an ordered two-way table by classifying the individuals and attributes, performed cluster analysis on the recovered isolates based on their response to biochemical tests. However, the scoring of bands for genetic diversity was 1 for presence and 0 for absence. The cluster analysis used the UPGMA method based on Jaccard's similarity coefficients with the SAHN module in NTSYS pc v 2.2 software (Rohlf, 2005).

RESULTS

Phenotypic evaluation

The recovered bacterial isolates totaling 19 from the root nodules of various leguminous plants collected from different sites recorded in Table 1. Most isolates were creamy or white opaque with moderate to heavy exopolysaccharide (EPS) production. The ability of isolates to absorb Congo red dye ranged from none to extremely weak. The colonies were round, mucoid, with a convex elevation, translucent, and smooth margined. Microscopic investigation revealed that all bacterial isolates are rod-shaped and have no positive affinity for Gram-stain. All isolates are motile.

Another intriguing and beneficial aspect of rhizobia is how they grow on the usual YEM medium, which uses bromothymol blue (BTB) as a pH indicator. Based on their growth rates on this common medium, the isolates showed varied growth patterns and abilities to produce acid and alkali (Table 4). On YEM with BTB plates, most rhizobial isolates exhibit an acid response and change medium color to yellow within three days of incubation at 28 °C. Dividing these rhizobial isolates can have two groups: those that multiply rapidly, demonstrating good growth within 24 to 48 h and forming large, vigorous colonies. However, 10 isolates formed colonies of 1–2 mm diameter after 5–7 days on the same medium and showed changing of medium pH to slight alkaline to alkaline (Table 4). These isolates come as slow-growth *Rhizobium*. Generally, the rhizobial strains grew adequately in two days converting the YEM agar medium containing bromothymol blue to yellow, indicating they were fast-growing acid producers with a mean of 24-h generation time. On the contrary, the slow-growing strains produced a yellowish-blue color in the medium, indicating they were slightly alkali producers with a generation time of 48–72 h.

Table 2. List of the primer names and their nucleotide sequences used in RAPD analysis.

No	Name	Sequence	No	Name	Sequence
1	OP-A3	5´CAG CAC CCA C 3`	4	OP-C15	5` GTG TGT GTG TGT TGT CC 3`
2	OP-A5	5´ CCTTGACGCA 3`	5	OP-D1	5´ ACC GCG AAG G 3`
3	OP-C9	5´ CTC ACC GTC C 3`	6	OP-K2	5` CAC GAG TCT C 3`

Table 3. List of the primer names and their nucleotide sequences used in ISSR analysis.

No	Name	Sequence	No	Name	Sequence
1	49A	5` CAC ACA CAC ACA AG 3`	4	HB-11	5` GTG TGT GTG TGT TGT CC 3`
2	HB-8	5` GAG AGA GAG AGA GG 3`	5	HB-12	5` CAC CAC CAC GC 3`
3	HB-10	5` GAG AGA GAG AGA CC 3`	6	HB-13	5´ GAG GAG GAG C 3`

Table 4. Characterization of rhizobial cultures and their growth pattern on differential media.

Isolate code	Growth Media								
	Glucose peptone			Keto-lactose agar		YMA-Congo red			
	Growth	Colony color	Medium color/pH	Growth	Colony color	Growth	Colony color	Growth rate	Colony size
1	-	-	-	±	Creamy	++	White	Fast	Large
2	+	Creamy	Purple/yellow	±	Creamy	+++	White	Fast	Large
3	+	Yellow	Yellow/acidic	±	Creamy	++++	Faint rosy white	Slow	Small
4	±	Yellow	Yellow/acidic	±	Creamy	++++	White	Fast	Large
5	-	-	Purple/Neutral	±	Creamy	+++	White	Fast	Large
6	±	Faint purple	Purple/Neutral	±	Creamy	++	Faint rosy white	Fast	Large
7	+	Yellow	Yellow/acidic	±	Creamy	+++	White	Slow	Small
8	+	Creamy	Purple/Neutral	±	Creamy	++	Faint rosy white	Slow	Small
9	-	-	-	±	Creamy	++	Faint rosy white	Fast	Large
10	+	Yellow	yellow acidic	±	Creamy	+++	White	Slow	small
11	+	Yellowish cream	Yellow/acidic	±	Creamy	++	Faint rosy white	Slow	small
12	+	Creamy	Purple/No change	±	Creamy	++	White transparent	Slow	Small
13	+	Yellowish cream	Purple/yellow	±	Creamy	+++	White	Fast	Large
14	±	change in color	Yellow/acidic	±	Creamy	+++	White	Slow	Small
15	-	-	-	-	Creamy	+++	Faint rosy white	Slow	Large
16	±	Creamy	Purple/No change	±	Creamy	++++	Faint rosy white	Fast	Large
17	+	Yellow	Yellow/acidic	+	Creamy	++++	White	Slow	Small
18	±	Yellowish cream	Yellow/slight acidic	±	Creamy	+++	White	Slow	Small
19	+	Creamy	Purple/Neutral	-	Creamy	++	White	Fast	Large

-, no growth; ±, highly poor growth; +, poor growth; ++, good growth; +++; excellent growth; ++++, heavy growth.

Fast and slow-growing *Rhizobium* spp. produced mucoid colonies, which were circular, convex with entire edges, and white in appearance. Furthermore, according to the confirmatory trial, most isolates grow poorly on glucose peptone medium. On the other hand, the poorly colonial growth on glucose peptone agar showed a creamy to a yellow colony with no change or slightly acidic medium. On the ketolactose agar, all isolates were negative for the formation of 3-ketolactose from lactose, and no yellow zone of Cu_2O was visible around the colonies after adding Benedict's reagent, which is a characteristic of *Rhizobium* cultures. Meanwhile, the growth on Hoffer's medium revealed none of the isolates were able to grow.

Biochemical characterization

For biochemical characterization, all *Rhizobium* isolates can produce catalase enzyme, indicated by the evolution of air bubbles when adding H_2O_2. For gelatin liquefaction, most isolates were unable to hydrolysis gelation except for isolates 7, 8, and 10 (recovered from root nodules of *Acacia saligna, Glycine max*, respectively). The citrate test showed that none of the isolates utilized citrate. Similar results emerged for H_2S production. The amylase production ability appeared on a modified YMA medium with the starch substituting the mannitol. All isolates showed a negative ability to hydrolyze starch except for isolates recovered from *Melilotus indicus* (L.), *Glycine max* (L.) Merr., *Cicer arietinum,* and *Trigonella foenum-graecum* (Isolates 1, 7, 8, 10, and 19, respectively). These isolates produced amylase and can break down starch (Table 5 and Figure 1A). Production of urease enzyme was detected in some isolates (5, 12, 13, 14, 16, and 18), which showed weak to strong urease production. (Table 5, Figure 1B). The strongest urease activity emerged for isolates 16 and 18 obtained from root nodules of *Lupinus termis - Forssk* and *Acacia saligna.* Fermentation-ability of some sugars revealed that all isolates could ferment mannitol, D-glucose, L-arabinose, and D-fructos.

Reinfection of the parent hosts

Following inoculation, all seedlings that served as the parent host for each *Rhizobium* strain started developing nodules along their root systems within two to four weeks. The number and strength of the nodules that developed on the inoculated seedling's root system varied depending on the *Rhizobium* culture applied; however, all isolates capably re-infected their hosts, proving that they are *Rhizobium* cultures.

Classification and diversity of rhizobial cultures

The agglomerative classification technique divided the examined rhizobial strains into two distinct groups based on their biochemical characteristics (Figure 2). Two sets of six and 13 isolates are generated for the first and second groups, respectively, by the created dendrogram. Subgroup A of the first group, which contains the rhizobial isolates 6, 7, 9, 14, and 18, was further separated. However, there is only one isolate in the second grouping B (isolate 11). In the meantime, the second group got divided into two more subgroups, which included subgroups C and D (Figure 2). Subgroup C contained three isolates: 2, 15, and 16. Subgroup D continued dividing into three different assemblies, with isolate 19 classed independently.

RAPD and ISSR analyses

The investigation of genetic divergence among 19 Egyptian rhizobial isolates used six RAPD and ISSR primers. For RAPD analysis, PCR exhibited 49 bands (Figures 3, 4, and Table 6) 42 of these were polymorphic (85.7%). The OP-A3 primer gave the highest number of polymorphic bands (nine), whereas the OP-D1 primer exhibited the lowest number (5). The highest similarity ratio occurred between isolates 18 and 19, with 89%, while the lowest ratio was between isolates 1 and 5, with 52%. The cluster analysis classified the isolates into two main groups (Figure 7). The first group included isolates 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 13, with the second group containing isolates 12, 14, 15, 16, 17, 18, and 19. In addition, ISSR primers produced 57 bands, with 55 of these (96%) polymorphic (Figures 5, 6, and Table 6). The registered maximum polymorphism levels were with primers HB-110, HB-11, HB-8, HB-12, HB-13, and 49 A. The HB-10 primer gave the highest number of polymorphic bands (11), with the 49 A primer having the lowest number (6). The highest similarity ratio came between isolates 1 and 4, with 83%, and the lowest among isolates 8 and 14 or 16, with a value of 39%. The phylogenetic tree divided the isolates into two clusters; the first cluster included isolates from 1 to 11, and the second group contained isolates from 12 to 19 (Figure 8).

Table 5. Biochemical characterization of *Rhizobium* cultures isolated from different leguminous plants located at different localities.

Biochemical characterization										
Isolate code	Catalase production	Gelatin liquefaction	Citrate utilization	H_2S production	Starch hydrolysis	Urease production	Sugar fermentation			
							Mannitol	D-glucose	L-arabinose	D-fructose
1	+	-	-	-	++	-	+	+	+	+
2	+	-	-	-	-	-	+	+	+	+
3	+	-	-	-	+	-	+	+	+	+
4	+	-	-	-	-	-	+	+	+	+
5	+	-	-	-	-	++	+	+	+	+
6	+	-	-	-	-	-	+	+	+	+
7	+	+	-	-	++	-	+	+	+	+
8	+	+	-	-	+++	-	+	+	+	+
9	+	-	-	-	-	-	+	+	+	+
10	+	+	-	-	++	-	+	+	+	+
11	+	-	-	-	-	-	+	+	+	+
12	+	-	-	-	++	+++	+	+	+	+
13	+	-	-	-	-	+	+	+	+	+
14	+	-	-	-	-	+++	+	+	+	+
15	+	-	-	-	+++		+	+	+	+
16	+	-	-	-	+	+	+	+	+	+
17	+	-	-	-	+	-	+	+	+	+
18	+	-	-	-	-	+	+	+	+	+
19	+	-	-	-	+++	-	+	+	+	+

‡ -, negative result; +, positive result; ++, moderate positive result; +++, strong positive result.

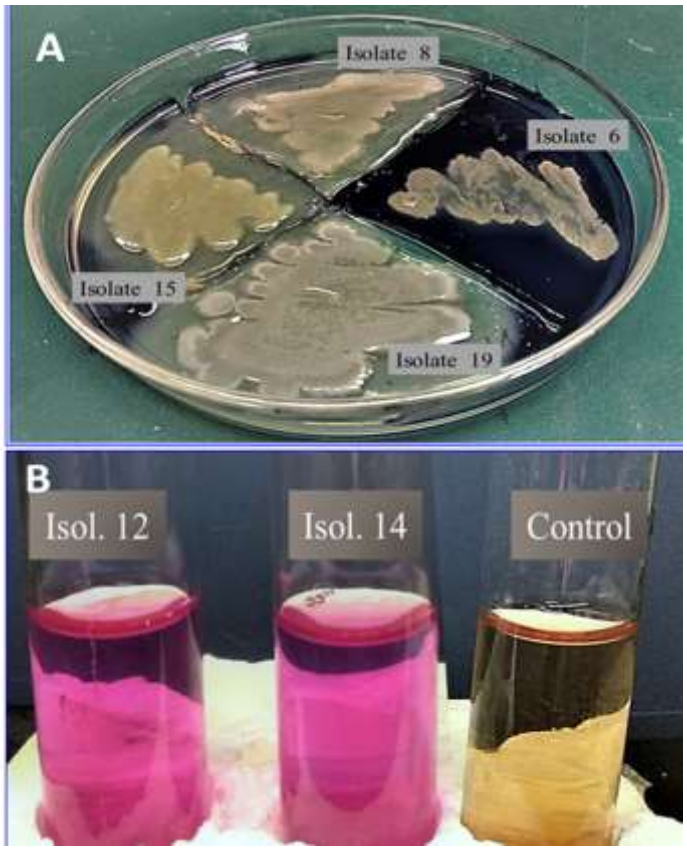

Figure 1. Production of amylase (A) and urease (B) by the tested isolates 12 and 14, as an example of a positive reaction.

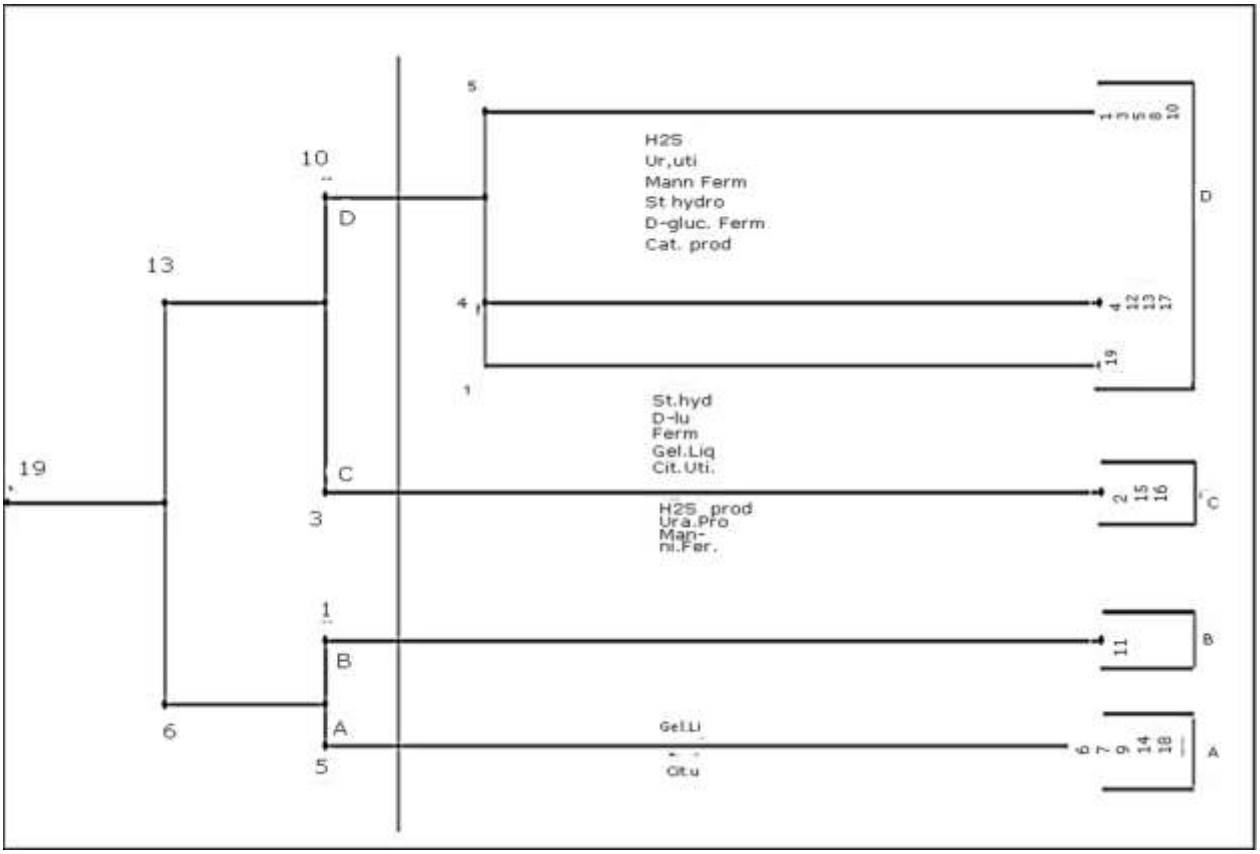

Figure 2. Classification of rhizobial isolates retrieved from different studied locations. A dendrogram developed using the agglomerative categorization method. The dendrogram generates two groups of isolates, which are then separated into four subgroups (A, B, C, and D) based on their biochemical reactions. The designation codes for the Rhizobial isolates are expressed by the written numbers.

Table 6. RAPD and ISSR analysis of *Rhizobium* isolates.

Primers	Band size (bp)	Total number of bands	Number of polymorphic bands	Polymorphic bands percentage (%)
OP-A3	200-1500	10	9	90
OP-A5	400-2200	8	7	87.5
OP-C9	200-1000	8	6	75
OP-C15	200-1200	9	8	88
OP-D1	400-2200	6	5	83
OP-K2	400-2200	8	7	87.5
Total		49	42	
49 A	300-1000	7	6	85.7
HB-8	200-1200	10	10	100
HB-10	200-1500	11	11	100
HB-11	200-1400	10	10	100
HB-12	200-1500	10	9	90
HB-13	200-1400	9	9	100
Total		57	55	

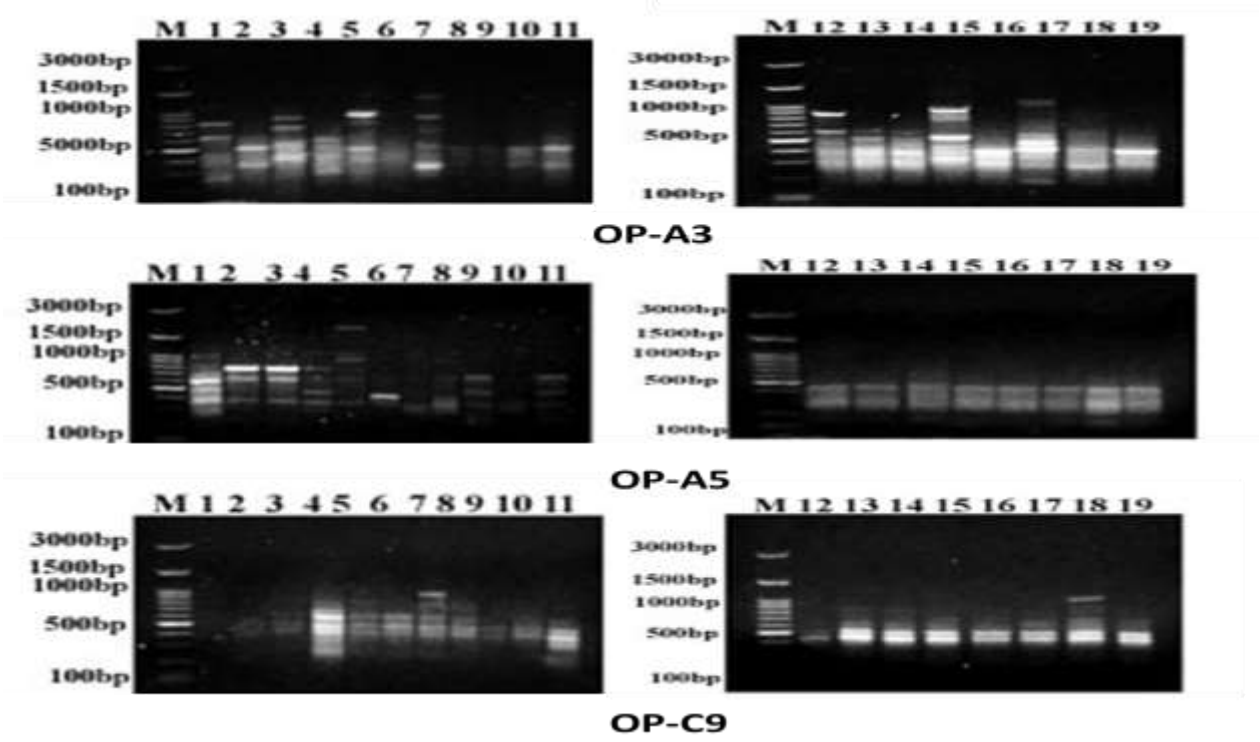

Figure 3. Genetic diversity among 19 rhizobial isolates (lanes 1-19) using RAPD primers (OP-A3, OP-A5 and OP-C9) and M: DNA ladder.

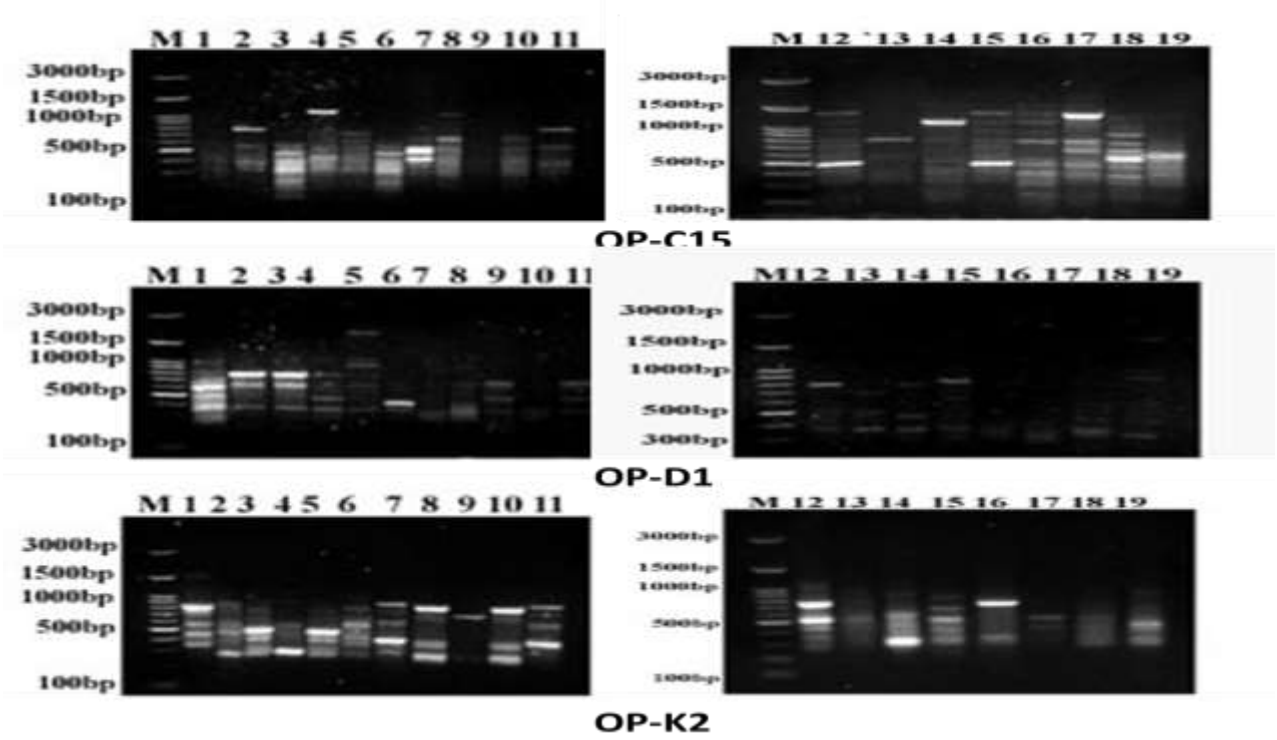

Figure 4. Genetic diversity among 19 rhizobial isolates (lanes 1-19) using RAPD primers (OP-C15, OP-D1, and OP-K2). M: DNA ladder.

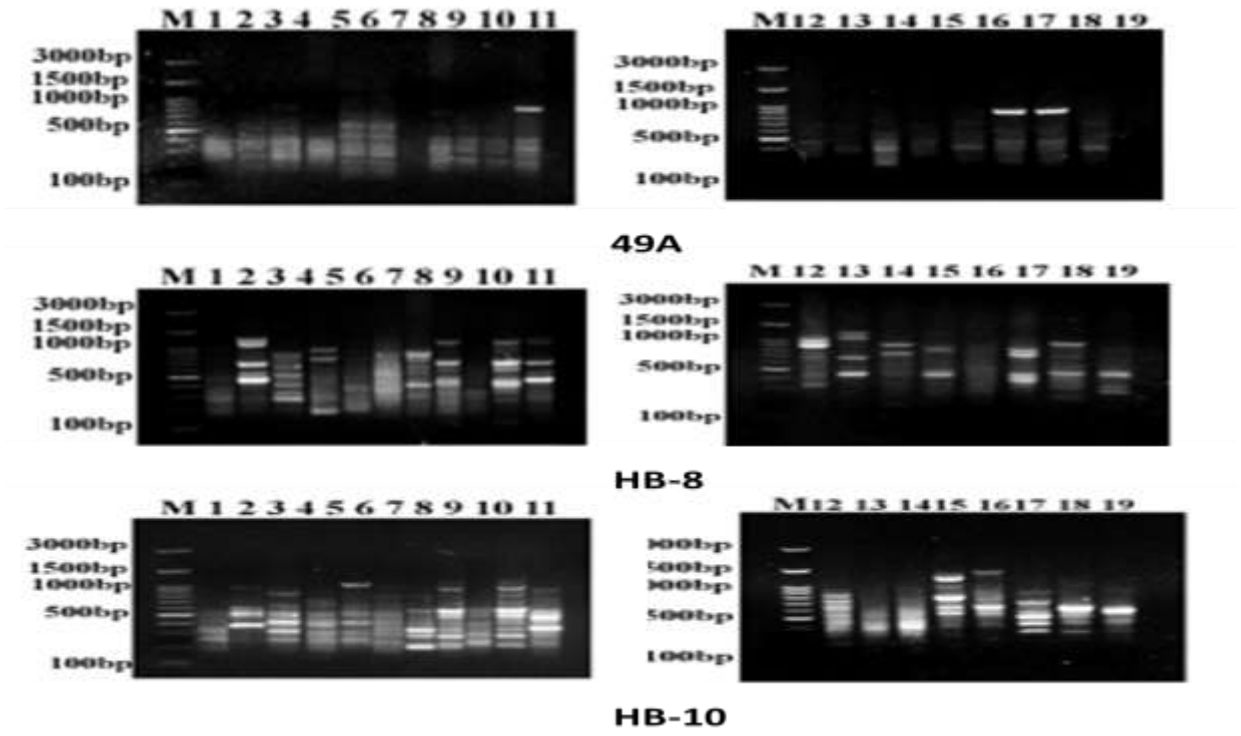

Figure 5. Genetic diversity among 19 rhizobial isolates (lanes 1-19) using ISSR primers (49A, HB-8, and HB-10), M: DNA ladder.

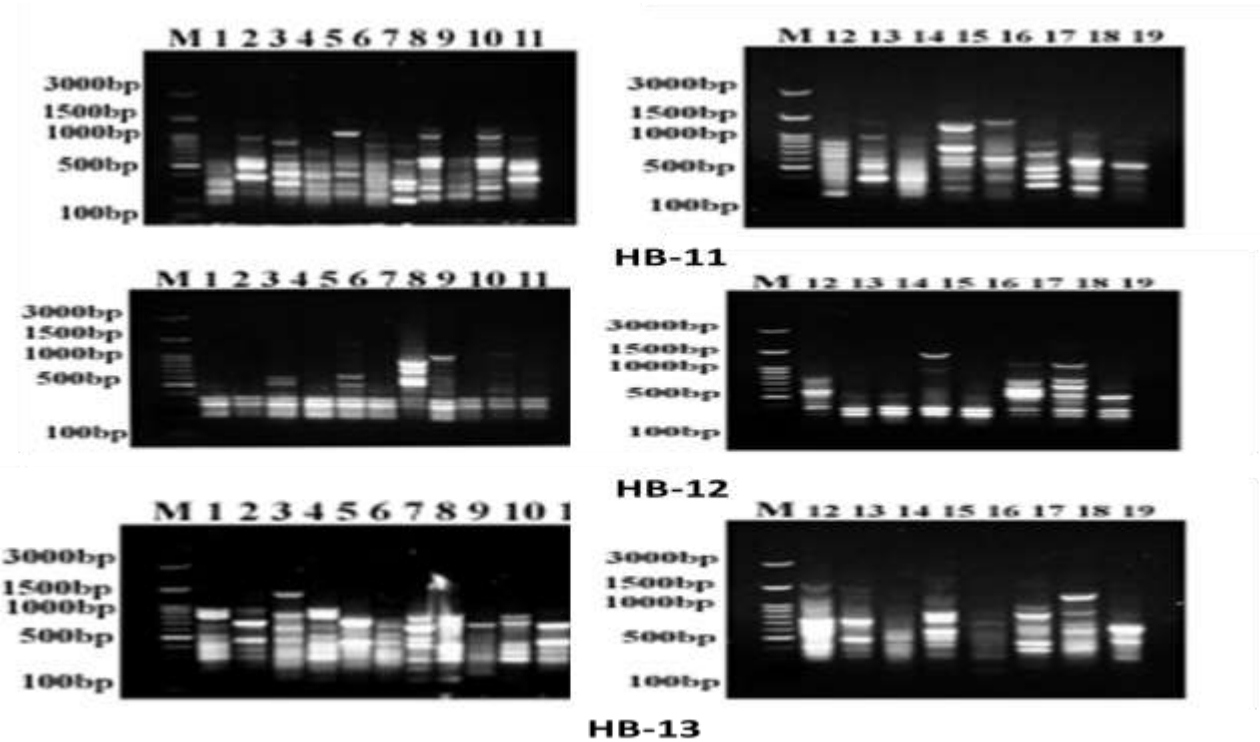

Figure 6. Genetic diversity among 19 rhizobial isolates (lanes 1-19) using ISSR primers (HB-11, HB-12, and HB-13), M: DNA ladder.

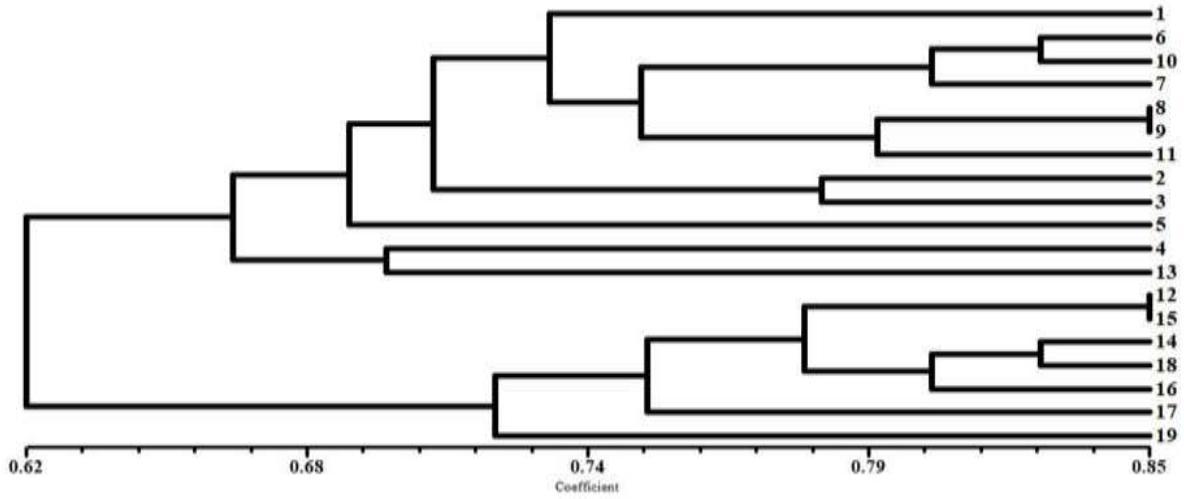

Figure 7. Dendrogram based on Jaccard's similarity coefficients scored from RAPD data using UPGMA algorithm.

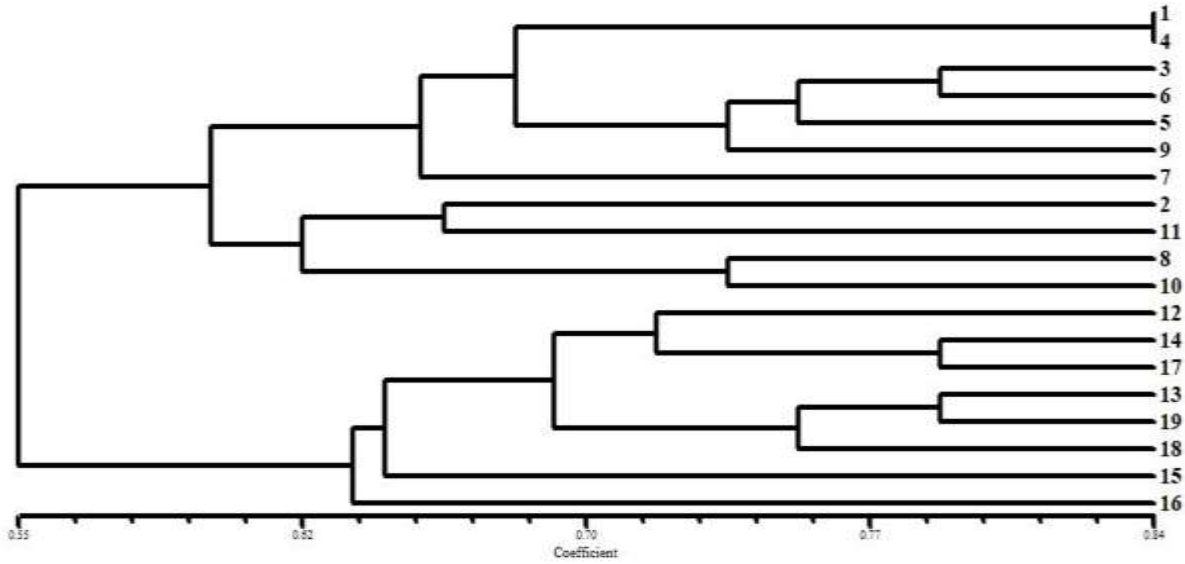

Figure 8. Dendrogram based on Jaccard's similarity coefficients scored from ISSR data using UPGMA algorithm.

DISCUSSION

This study recovered 19 isolates of rhizobia from root nodules of different legume plants, such as, clover, fenugreek, broad bean, groundnuts, soybean, and chickpea collected from various sites in Ismailia governorate, Egypt. Identification of these isolates as rhizobia cultures included the colony morphology and its reaction with Congo red dye, as well as, their ability to re-infect their parent host. Microscopic investigation revealed that all bacterial isolates are rod-shaped and have no positive affinity for Gram-stain. Previous studies showed that rhizobia could not absorb Congo red dye as the discriminatory character of rhizobia, with negative-Gram rod cells with motile ability (Singha *et al.*, 2015; Gilbert *et al*., 2018; Samuel *et al*., 2021).

Regarding the growth pattern of all the isolates evaluated on diverse differential media—YEM medium, glucose peptone, and ketolactose agar—all cultures showed confirmatory positive results for *Rhizobium* strains identified in another study (Kumar *et al*., 2020). Poor growth in glucose peptone medium after two days of inoculation also occurred in studies done by Upadhayay *et al.* (2017), which agrees with the present results. For growth on YEM medium, isolates classification could be fast- and slow-growing rhizobia. These results align with the previous study that revealed the existence of each fast- and slow-growing rhizobia in different soils (Zhang *et al*., 1991).

Concerning biochemical characterization, most isolates showed typical rhizobia culture characterization, as seen in a previous study by Panwar *et al.* (2013), in which all isolates generated catalase enzyme. Based on the latest results, the isolates' capacity to liquefy gelatin appears weak. The isolates have a limited ability to dissolve gelatin. This result contradicts a study by Mohammed and Sultan (2021); they reported that most rhizobial local strains showed positive results for the gelatin liquefaction test. Kumari *et al.* (2010) also observed diverse outcomes with different rhizobial isolates, mentioning that the gelatinase enzyme plays a vital role during nodule formation.

Citrate utilization by *Rhizobium* cultures showed variation among cultures, as recorded in an earlier study (Panwar *et al*., 2013). However, in this study, all isolated *Rhizobium* cultures showed an adverse ability to utilize citrate. This result agrees with the study by Hamza and Alebejo (2017). Furthermore, *Rhizobium* cultures could not produce H_2S, although H_2S is considered a gaseous signal molecule that stimulates nodule development and, as a result, regulates plant growth (Zou *et al*., 2019).

Investigating hydrolysis of either starch or urea showed diverse groups of *Rhizobium* cultures in their capabilities for α-amylase and urease production (Kamthane, 2021). Native isolates from selected sites in this study showed variation in potentiality for the breakdown of starch or urea as a sole carbon or nitrogen source, respectively. Study results agree with studies that tested *Rhizobium* cultures for enzyme production like α-amylase and urease (Kamthane, 2021).

DNA markers, such as ISSR and RAPD, successfully revealed the genetic divergence among the tested isolates. Generally, the ISSR marker proved a more powerful tool in discriminating among the tested isolates than the RAPD marker. The results showed some variance in the cluster analysis between RAPD and ISSR markers. These differences are accepted because each marker targets various genome regions. A previous study reported that not all accessions attached to the same variety are classified in the same category (Xiong *et al*., 2011). Moreover, RAPD and ISSR markers, employed previously, explored the genetic variability among several wheat varieties and proved that the ISSR markers gave more frequency (El-Assal and Gaber, 2012). Fathy *et al.* (2021) used the ISSR marker to study the genetic profiling of five rhizobial isolates. PCR produced 37 genomic loci, 54% of them polymorphic. They concluded that clustering analysis based on molecular data was valuable in selecting promising rhizobial isolates for biological nitrogen fixation. Finally, previous studies employed RAPD fingerprinting to estimate the diversity and establish a genetic relationship between fast-growing rhizobia (Ghosh *et al*., 2017; Flores-Félix *et al*., 2020).

CONCLUSION

The first-time application of the agglomerative classification technique (TWINSPAN) in this study demonstrated the biochemical characterizations of all examined strains that can be a suitable, valuable, distinct, and accessible tool for diversity among tested rhizobial strains. Therefore, performing extra biochemical tests and applying the TWINSPAN classification tool is a high recommendation, in combination with the molecular technique, to clarify the diversity among rhizobial cultures.

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REFERENCES

Abdel-Lateif KS, Abd El-Ghany EM (2023). Characterization of Rhizobium isolates as biocontrol agents against Fusarium solani and Macrophomina phaseolina. *SABRAO J. Breed. Genet*. 55(1): 146-155. http://doi.org/10.54910/sabrao2023.55.1.14.

Al-Kurtany AES, Ali SAM, Oleawy MF (2023). Tomato seedling production using an inoculum prepared with plant growth-promoting rhizobacteria (pgpr) isolates *SABRAO J. Breed. Genet*. 55(1): 230-236. http://doi.org/10.54910/sabrao2023.55.1.21.

Bhuyan SI, Hossain MS, Islam MM, Begum SN, *et al.* (2014). Molecular assessment of genetic diversity and relationship in selected green gram germplasms. *Biotechnol*. 13:126-134. doi: 10.3923/biotech.2014.126.134.

Daiwshala CK (2021). Starch hydrolysis by *Rhizobium japonicum* species from different soybean varieties. *Int. J. Adv. Biotechnol. Res*. 12(4): 1-8.

El-Assal SD, Gaber A (2012). Discrimination capacity of RAPD, ISSR, and SSR markers and of their effectiveness in establishing genetic relationship and diversity among Egyptian and Saudi wheat cultivars. *Am. J. Appl. Sci.* 9: 724-735.

Elavarasi P, Yuvaraj M, Gayathri P (2020). Application of bacteria as a prominent source of biofertilizers'. in SM Mirmajlessi, R Radhakrishnan (eds.), *Biostimulants in Plant Science*, Intech Open, London.

Etminan A, Pour-Aboughadareh A, Mohammadi R, Ahmadi-Rad A, Noori A, Mahdavian Z, *et al.* (2016). Applicability of start codon targeted (SCoT) and inter-simple sequence repeat (ISSR) markers for genetic diversity analysis in durum wheat genotypes. *Biotechnol. Biotechnol. Equip*. 30:1075-1081. https://doi.org/10.1080/13102818.2016.1228478

Fathy DM, Eldomiaty AS, Abd El-Fattah HI, Mahgoub EI, Hassanin AA (2021). Genetic profiling and diversity of some promising efficient rhizobial isolates on *Faba Bean* plants. *Zagazig. J. Agric. Res*. 48:2. doi:10.21608/ZJAR.2021.175279.

Fetyan NA, Mansour AA (2012). Molecular, biochemical, and physiological characterization of symbiotic bacteria isolated from saline soil in Saudi Arabia. *Aust. J. Basic. Appl. Sci.* 6: 229-239.

Flores-Félix JD, Carro L, Cerda-Castillo E, Squartini A, Rivas R, Velázquez E (2020). Analysis of the interaction between *Pisum sativum* L. and *Rhizobium laguerreae* strains nodulating this legume in Northwest Spain. *Plants* 9:1755. https://doi.org/10.3390/plants9121755

Ghosh M, Sharma P, Sirari A, Kaur N, Chand K (2017). Phenotypic and genotypic analysis of summer *Mungbean Rhizobia*. *Int. J. Curr. Microbiol. Appl. Sci.* 6(9):3149-3162. https://doi.org/10.20546/ijcmas.2017.609.389.

Gilbert, Mburu Simon W, Kimiti Jacinta M, Ombori Omwoyo, Maingi John M, Njeru Ezekiel M (2018). Genetic characterization and diversity of rhizobium isolated from root nodules of mid-altitude climbing bean (*Phaseolus vulgaris* L.) varieties. *Front Microbiol.* 9: 968. https://www.frontiersin.org/articles/10.3389/fmicb.2018.00968.

Hakim S, Mirza BS, Imran A, Zaheer A, Yasmin S *et al.* (2020). Illumina sequencing of 16S rRNA tag shows the disparity in rhizobial and non-rhizobial diversity associated with root nodules of mung bean (*Vigna radiate* L.) growing in different habitats in Pakistan. *Microbiol. Res.* 231: 126356. DOI: 10.1016/j.micres.2019.126356.

Hamza TA, Alebejo AL (2017). Isolation and characterization of *rhizobia* from rhizospher and root nodule of cowpea, elephant, and lab plants. *Int. J. Nov. Res. Interdiscip. Stud.* 4: 1-7. https://doi.org/10.1111/pbr.12057.

Hill, MO (1979). TWINSPAN: A FORTRAN program for arranging multivariate data in an ordered two-way table by classification of the individuals and attributes. Ithaca, New York. USA.

Hussein E, Mohamed A, Attia S, Adawy S (2006). Molecular characterization and genetic relationships among cotton genotypes 1-RAPD, ISSR, and SSR analysis. *Arab J. Biotechnol.* 9: 313-328.

Jaiswal SK, Mohammed M, Ibny FYI, Dakora FD (2021). *Rhizobia* as a source of plant growth-promoting molecules: Potential applications and possible operational mechanisms. *Front. Sustain. Food. Syst*. 4: 619676. doi:10.3389/fsufs.2020.619676.

Khalid R, Zhang XX, Hayat R, Ahmed M (2020). Molecular characteristics of rhizobia isolated from *Arachis hypogaea* grown under stress. *Environ. Sustain.* 12:6259. https://doi.org/10.3390/su12156259.

Kumar J, Ramlal A, Mallick D, Mishra V (2021). An overview of some biopesticides and their importance in plant protection for commercial acceptance. *Plants* 10:1185. doi: 10.3390/plants10061185.

Kumar KB, Mir I, Hameeda B (2020). Morphological, biochemical, and intrinsic antibiotic resistance of rhizobia isolated from root and stem nodules of various leguminous plants. *Plant Cell. Biotechnol. Mol. Biol*. 21: 126-138.

Kumari BS, Ram MR, Mallaiah KV (2010). Studies on nodulation, biochemical analysis, and protein profiles of Rhizobium isolated from Indigofera species. *Malaysian J. Microbiol.* 6(2): 133-139. doi:10.21161/MJM.20109.

Mansour SR, Zayed A, Safwat M (2003). N2-fixation efficiency of *Acacia saligna* inoculated with *Bradyrhizobium* strains under field

conditions. *Int. J. For. Usufructs Manage.* 4:22-27.

Mohammed AA, Sultan RH (2021). Isolation and characterization of local isolates of *Rhizobia* in Nineveh governorate/Iraq. *J. Edu. Sci.* 30(3): 2-22. 10.33899/edusj.2021.168657.

Nelson LM, Child JJ (1981) Nitrogen fixation and hydrogen metabolism by *Rhizobium leguminosarum* isolates in pea root nodules. *Can. J. Microbiol*. 27:1028-1034.

Panwar A, Choudhary S, Sharma M, Sharma Y, Meena RS, Malhotra S, *et al.* (2013). Morphological and biochemical characterization of *Rhizobium* isolates obtained from fenugreek (*Trigonella foenum*). *Seed. Res*. 40(2): 196-200.

Peoples MB, Herridge DF, Ladha JK (1995). Biological nitrogen fixation: An ancient source of nitrogen for sustainable agricultural production. In Management of Biological Nitrogen Fixation for the Development of More Productive and Sustainable Agricultural Systems; Springer: Berlin, Germany. 3-28.

Rai R, Dash PK, Mohapatra T, Singh A (2012). Phenotypic and molecular characterization of indigenous rhizobia nodulating chickpea in India. *Indian. J. Exp. Biol.* 50: 340-350.

Rajasundari K, Ilamurugu K, Logeshwaran P (2009). Genetic diversity in rhizobial isolates determined by RAPDs. *Afr. J. Biotechnol*. 8(12): 2677-2681.

Randhawa HS, Pozniak CJ, Hucl PJ, Knox RE, McCartney C, Spaner DM (2013). Application of molecular markers to wheat breeding in Canada. *Plant Breed.* 132: 458-471.

Rohlf F (2005). Numerical Taxonomy and Multivariate Analysis System Version 2.2 Exeter Software. New York, USA.

Ruiu L (2018). Microbial biopesticides in agroecosystems. *Agron.* 8: 235. https://doi.org/10.3390/ agronomy 8110235.

Samuel TN, Poole PH, Sánchez-Cañizares C (2021). Rhizobial chemotaxis and motility systems at work in the soil. *Front. Plant. Sci.* 12: 725338. https://doi.org/10.3389/fpls. 2021.725338.

Shoukry AA, Khattab AA, Abou-Ellail M, El-shabrawy H (2013). Molecular and biochemical characterization of new *Rhizobium leguminosarum* bio *viciae* strains isolated from different locations of Egypt. *J. Appl. Sci. Res.* 9(11): 5864-5877.

Singha B, Das P, Mazumder P (2015). Morphological and biochemical characterization of rhizobia isolated from root nodule of *Crotolaria junceae* L. grown in Assam. *Int. J. of Sci. and Res*. pp. 438.

Torrey JG (1978). Nitrogen fixation by actinomycete-nodulated angiosperms. *BioSci.* 28(9): 586-592.

Upadhayay S, Pareek N, Mishra G (2017). Short Communication: Isolation and biochemical characterization of *Rhizobium* strains from nodules of lentil and pea in Tarai agro-ecosystem, Pantnagar, *Indian J. Nasional.* 7(2): 73-76. doi:10.13057/nusbiosci/ n070203.

Wagner SC (2011). Biological nitrogen fixation. *Nat. Educ. Know*. 3(10):15.

Williams JGK, Kubelk AR, Livak KJ, Rafalski JA, Tingey S-V (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nuc. Acid. Res.* 18: 6231-6235.

Xiong F, Zhong R, Han Z, Jiang J, He L, Zhuang W, *et al*. (2011). Start codon-targeted polymorphism for evaluation of functional genetic variation and relationships in cultivated peanut (*Arachis hypogaea* L.) genotypes. *Mol. Biol. Rep.* 38: 3487-3494. doi: 10.1007/s11033-010-0459-6.

Zhang X, Harper R, Karsisto M, Lindstrom K (1991). Diversity of *Rhizobium* bacteria isolated from the root nodules of leguminous trees. *Int. J. Syst. Bacteriol*. 41(1): 104-113.

Zhang Y, Yan H, Jiang X, Wang X, Huang L, Xu B, *et al*. (2016). Genetic variation, population structure, and linkage disequilibrium in Switchgrass with ISSR, SCoT, and EST-SSR markers. *Hereditas.* 153:4.

Zou H, Zhang NN, Pan Q, Zhang JH, Chen J, Wei GH (2019). Hydrogen sulfide promotes nodulation and nitrogen fixation in the soybean-rhizobia symbiotic system. *Mol. Plant Microbiol. Interact.* 32(8): 972-985. doi: 10.1094/MPMI-01-19-0003-R.