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GENETIC PROFILING AND DIVERSITY OF SOME PROMISING EFFICIENT RHIZOBIAL ISOLATES ON FABA BEAN PLANTS

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ABSTRACT: A group of twenty faba bean rhizobial isolates was collected from two Egyptian Governorates (Dakahlia and Damitta). The isolates were further morphologically and physiologically characterized to check their growth and symbiotic performance on faba bean plants. According to remarkable lab and pots tests, five rhizobial isolates (Rh 32, Rh 6-A, Rh 3-4, Rh RL3, and Rh 8-A) were selected and subjected to further biochemical and molecular characterizations. Genetic profiling of the five promising rhizobial isolates was conducted using six ISSR-primers. Amplification of bacterial genomic DNA produced a total of 37 genomic loci, 54% of them were polymorphic and 46% were monomorphic. The rate of polymorphism ranged between 25% to 80% with an average of 54%. Clustering pattern analysis of morphological and physiological data grouped the twenty rhizobial isolates in five clusters and the five selected rhizobial isolates were falling close to each other. Clustering analysis of ISSR data grouped the the five rhizobial isolates in four clusters. Analysis based on ISSR data revealed that the lowest genetic distance was 2.00 between Rh 6-A and Rh 3-4 isolates, while the highest genetic distance of 3.61 was between Rh 32 and each of Rh 6-A, Rh 3-4, and Rh RL3 isolates. The greatest similarity measurement was 0.931 between Rh 6-A and Rh 3-4 isolates; while the lowest similarity was 0.745 between Rh 32 and Rh 3-4 isolates. It can concluded that clustering pattern analysis based on molecular data could be used in facilitating the selection of rhizobial isolates that will be promising as a source of genes for biological nitrogen fixation and plant growth-promotion.

Key words: Faba bean, genetic profiling, rhizobia, ISSR, clustering analysis.

INTRODUCTION

Microbial biodiversity forms a huge natural resource for agriculturists. Root-nodulating bacteria (rhizobia) are particularly useful due to their symbiotic nitrogen fixing ability in symbiosis with legume plants and it more relevant for sustainable agriculture worldwide (**Modigan *et al.*, 2015**). It plays a major role in sustaining soil health for crop production. The diversity of faba bean-nodulating rhizobia has been widely investigated using molecular techniques, which include the use of PCR-based methods to characterize the isolates (**Wolfe and Liston, 1998; Mort *et al.*, 2003; Hettwer and Gerowitt, 2004**).

Inter Sequence Simple Repeat (ISSR) analysis using the polymerase chain reaction (PCR) provides a simplified method for characterization and fingerprinting of rhizobial isolates at the molecular level (**Neves and Rumjanek, 1997**).

Phylogenetics and clustering analyses estimated based on PCR-ISSR and morphological data provide important insights into the selection and taxonomy of organisms relying on their attributes. ISSR markers are based on microsatellites through the designing of primers to amplify the regions that are genomically located between microsatellite regions. This achieves a fingerprint and determines the genetic diversity from single primer PCR (**Wolfe and Liston, 1998; Hettwer and Gerowitt, 2004**).

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Legumes based on area harvested (190 million hectares) and total production (300 million tons) worldwide are considered the second most important crop (**Getachew, 2019**). The cultivated areas and yields of faba bean plants in Egypt were 27824/ ha (ha= 2.38 fed.) 95789 Tons **(FAO, 2019)**.Through symbiosis with *Rhizobium* bacteria, Legumes can fix the atmospheric nitrogen in the soil, thereby reducing the need for high prices chemical fertilizers, and to serve as rotation crops is very important to current systems of agricultural production (**Lakhani *et al.*, 2017**). Nodules and rhizospheric rhizobia are known for their potential for nitrogen fixation in the legumes crops (**Doyle and Luckow, 2003**). The difference in the ability of rhizobia to fix atmospheric nitrogen up to 450 Kg N/ha is due to the genetic diversity between rhizobium bacterial strains (**Stephens and Rask, 2000**).

Faba bean (*Vicia faba* L.) is a fundamental economic crop, so it is a key aspect to collect and characterize the rhizobia associated with this plant species in Egypt.

In the current research, the five selected rhizobial isolates were investigated using ISSR-PCR technique to determine and understand the genetic diversity of promising candidates for inoculants development. This work further intends to determine the usefulness of genetic fingerprinting and clustering analysis in profiling and selecting featured efficient rhizobial isolates.

MATERIALS AND METHODS

Twenty rhizobial isolates were screened to select the more efficient rhizobial isolates for their ability to form root nodules on faba bean plants. Five rhizobial isolates (Rh 32, Rh 6-A, Rh 3-4, Rh RL3, and Rh 8-A) out of the 20 isolates were selected on the basis of their remarkable growth biomass determination in minimal medium (**Beringer, 1974**), and in complete medium (**Somasegaran and Hoben, 1994**) as well as their ability to produce IAA (**MacFaddin, 2000; Hemraj *et al.*, 2013**), indole-acetic acid production, roots weight (gm/ plant) and nodulation properties. The present investigation was conducted in laboratories of Faculty of Agriculture Zagazig University, as well as in a pot experiment at the Research Station of TAG El-Aze, Agricultural Research Center Egypt.

Isolation and Purification of Rhizobia

True pink ridsh-root nodules were carefully collected from faba bean plants grown in two different Governorates of Egypt (Al Dakahlia and Damietta). The collected samples were put in sterile paper bags and transferred immediately for the isolation process. Rhizobial bacteria were isolated from nodules extracts (**Vincent, 1970**) and soil samples dilutions (**Somasegaran and Hoben, 1994**) using streak and pour-plate methods, respectively.

Single rhizobial colonies that appeared on yeast extract mannitol agar (YEMA) plates within 3- 5 days were picked up and cultured on YEMA slants, isolates was purified again by repeated streak plating for 3 times.

To differentiate between the isolated rhizobial from closely related soil bacteria, Congo red test (0.025 g/L) into YEMA plates (**Vincent, 1970**) and bromo creasol purple (0.005 g/L) into peptone glucose medium (**Somasegaran and Hoben, 1994)** were performed to distinct rhizobia from *Agrobacterium* and *Bradyrhizobium*, respectively.

Nodulation Ability of Rhizobial Isolates in Pots Experiment

The nodulation ability experiment was conducted to compare the effctivness of the five rhizobia isolates on nodulation properties of faba bean plants (variety Giza 843). The experiment was designed in a Randomized Complete Block Design (RCBD) with three replicates including the five rhizobial isolates and the uninoculated control plants. Faba bean seeds were surface sterilized, mixed with sterilized peat- based inoculants containing 10^8 CFU/ml of previously prepared rhizobial cultures according to the standards procedures of **Somasegaran and Hoben (1994)**. The coated seeds were planted; all the agronomical practices of faba bean production were applied. During the flowering stage (55 days after seed sowing), three plants of each block were randomly harvested for determining of

nodulation properties (number of nodules/ plant, weight of nodules/plant) (**Somasegaran and Hoben, 1994**).

ISSR-PCR Amplification

Six ISSR primers (Table 1) were used to amplify genomic DNA from the five rhizobial isolates. PCR reaction was performed in a total volume of 25 µL containing 3 µL of DNA (40 ng/ µL), 1.5 µL of the ISSR primer (10 µM), 2.5 µL of 10X reaction buffer, 2 units of Taq DNA polymerase (5 U/µL), 0.5 mM dNTPs, and 5 mM MgCl2. PCR amplifications were performed in a thermal cycler (Applied Biosystems, USA) programmed as 95°C for 5 min for an initial denaturation, 30 cycles of denaturation at 95°C for 30s, annealing temperature as mentioned in Table 1 for 30s, and 72°C for 2 min of extension; a final extension at 72°C for 5 min.

Electrophoresis of PCR Products

ISSR-PCR products were visualized by 1.5% agarose gel stained with ethidium bromide in TBE buffer (pH 8.5) for 20 min. 1.5 kbp DNA ladder was used as molecular size standard and the products were finally photographed using the gel documentation system (WVP, USA).

Clustering Pattern Analysis

The ISSR-based PCR band fragments were scored as present (1) or absent (0). Genetic diversity was estimated by comparing the banding patterns of the five isolates. The polymorphism rate was estimated by dividing the polymorphic genomic loci by the total number of scored loci. 1.5kbp ladder (Invitrogen, USA) was used to estimate band size. Genetic similarity among genotypes was calculated according to Dice measurement (**Dice, 1945**) using IBM SPSS statistics program (**Norušis, 1993**). The clustering analysis (**Rokach, 2005**) was applied to grouping and generating the linkage dendrogram using STATISTICA 8 software (**Weiß, 2007**).

RESULTS

Morphological and Physiological Properties of Rhizobial Isolates

Morphological characterization, biomass density (at 620 nm), roots weight (gm/plant), and IAA production (mg/mL) for the twenty rhizobial isolates were performed. All these characterizations were recorded according to (**Palaniappan *et al.*, 2010**). The data revealed that five isolates (Rh 32, Rh 6-A, Rh 3-4, Rh RL3 and Rh, 8-A) out of the 20 isolates were distinguished from the rest of isolates and seemed to be promising in their nitrogen-fixing parameters (Table 2).

Molecular Characterization Analyses

Rhizobial isolates that showed remarkable morphological and biochemical characteristics were subjected to further molecular fingerprinting analyses. PCR-ISSR based analysis reveled that the data for five selected rhizobial isolates using six ISSR primers showed a total of 37 genomic loci, 54% of them were polymorphic and 46% were monomorphic, the average number of amplified genomic loci was 6.16 genomic loci per primer (Table 3). All primers generated reliable polymorphic genomic loci with all rhizobial isolates (Fig. 1). The ISSR primers achieved a rate of polymorphism ranged between 25% to 80% (Table 3) which successfully explains that ISSR primers are efficient in determining the genetic fingerprinting and discrimination of rhizobial isolates (**Arora *et al.*, 2018**) due to their high ability to produce polymorphic loci.

Genetic Distance and Similarities

Both of genetic distances and genetic similarities were determined for the five promising rhizobial isolates using ISSR-PCR markers, the greatest similarity was 0.931 between Rh 6-A and Rh 3-4 isolates, while the lowest similarity was 0.745 between Rh 32 and Rh 3-4 isolates (Table 4). The minimum genetic distance was 2.00 between Rh 6-A and Rh 3-4 isolates, while the highest genetic distance of 3.61 was between Rh 32 and each of Rh 6-A, Rh 3-4, and Rh RL3 isolates (Table 5).

Clustering Pattern Analysis

The clustering analysis of the twenty bacterial isolates based on the morphological and physiological attributes grouped them into five distinct groups (Fig. 2). Each of Rh 4-5 and Rh 32 formed independent clusters (I) and (ш), respectively. Rh 3-7, Rh Rl3, Rh 8-A, and Rh 3-2 isolates formed cluster (IV). The rest of rhizobial isolates formed cluster (V). On the other hand, based on the ISSR data, clustering analysis divided the five bacterial isolates into three groups (Fig. 3). Both of Rh RL3, Rh 8-A

Table 1. ISSR primers with their respective sequences, GC percentage, annealing temperatures, and primer length

Primer	Sequence of nucleotides (5 - 3)	GC (%)	Annealing temperature (°C)	Primer length
ISSR 1	AGAGAGAGAGAGAGAGYC	56	60	18
ISSR 2	GAGAGAGAGAGAGAGATT	44	55	18
ISSR 3	AGAGAGAGAGAGAGAGC	53	57	17
ISSR 4	GAGAGAGAGAGAGAGC	53	57	16
ISSR 5	GAAGAAGAAGAAGAAGAA	33	51	18
ISSR 6	CACACACACACACACARY	44	55	18

R: purine (A or G), Y: pyrimidine (C or T)

Table 2. Mean performance of the 20 rhizobial isolates with regard to growth density in complete and minimal media, indole acetic acid (mg/ml) production, roots weight (gm/ plant), and nodulation status

Isolates		Biomass density (620nm)		IAA production (mg/ mL)	Roots weight (gm/ plant)	Nodulation status	
No	**Code**	**Complete**	**Minimal**			**NO./ plant**	**Weight (gm/plant)**
1	**Rh 4-4**	0.78	0.74	0.46	12.97	17	0.57
2	**Rh 3-2**	0.45	0.36	0.21	22.57	28	0.94
3	**Rh 3-1**	0.73	0.69	0.22	20.96	16	0.90
4	**Rh 3-3**	0.79	0.57	0.18	22.00	11	0.58
5	**Rh 8-A**	1.83	1.76	1.38	22.57	31	0.92
6	**Rh RL3**	1.99	1.87	1.41	22.82	31	0.94
7	**Rh 22-A**	1.94	0.77	0.05	18.37	12	0.55
8	**Rh 3-4**	2.04	1.95	1.56	24.63	43	1.14
9	**Rh 4-5**	1.16	1.06	0.04	8.55	6	0.39
10	**Rh 4-3**	0.51	0.41	0.050	15.67	18	0.73
11	**Rh 31**	0.82	0.78	0.18	19.17	14	0.61
12	**Rh 18-A**	0.49	0.38	0.07	22.14	16	0.52
13	**Rh 3-7**	1.65	1.46	1.10	14.16	26	0.92
14	**Rh 32**	2.10	2.05	1.77	32.35	124	1.70
15	**Rh 9-A**	0.30	0.16	0.81	20.39	16	0.89
16	**Rh 6-A**	2.00	1.98	1.59	25.92	48	1.15
17	**Rh 21-A**	0.77	0.61	0.06	20.12	10	0.52
18	**Rh 4-2**	1.02	0.85	1.01	19.13	11	0.45
19	**Rh 4-6**	0.80	0.63	0.11	18.33	14	0.59
20	**Rh RL**	0.50	0.17	1.09	19.9	12	0.49

Table 3. Total numbers of loci, number of monomorphic loci, number of polymorphic loci, number of unique loci, and rate of polymorphism of six ISSR primers in some rhizobial isolates

Primer	Number of loci	Monomorphic loci	Polymorphic loci	Unique loci	Rate of polymorphism (%)
ISSR-1	5	1	4	1	80
ISSR-2	8	6	2	0	25
ISSR-3	7	3	4	1	57
ISSR-4	4	3	1	0	25
ISSR-5	6	2	4	0	66
ISSR-6	7	2	5	1	71
Average	6.16	2.83	3.33	0.5	54
Total	37	17	20	3	-

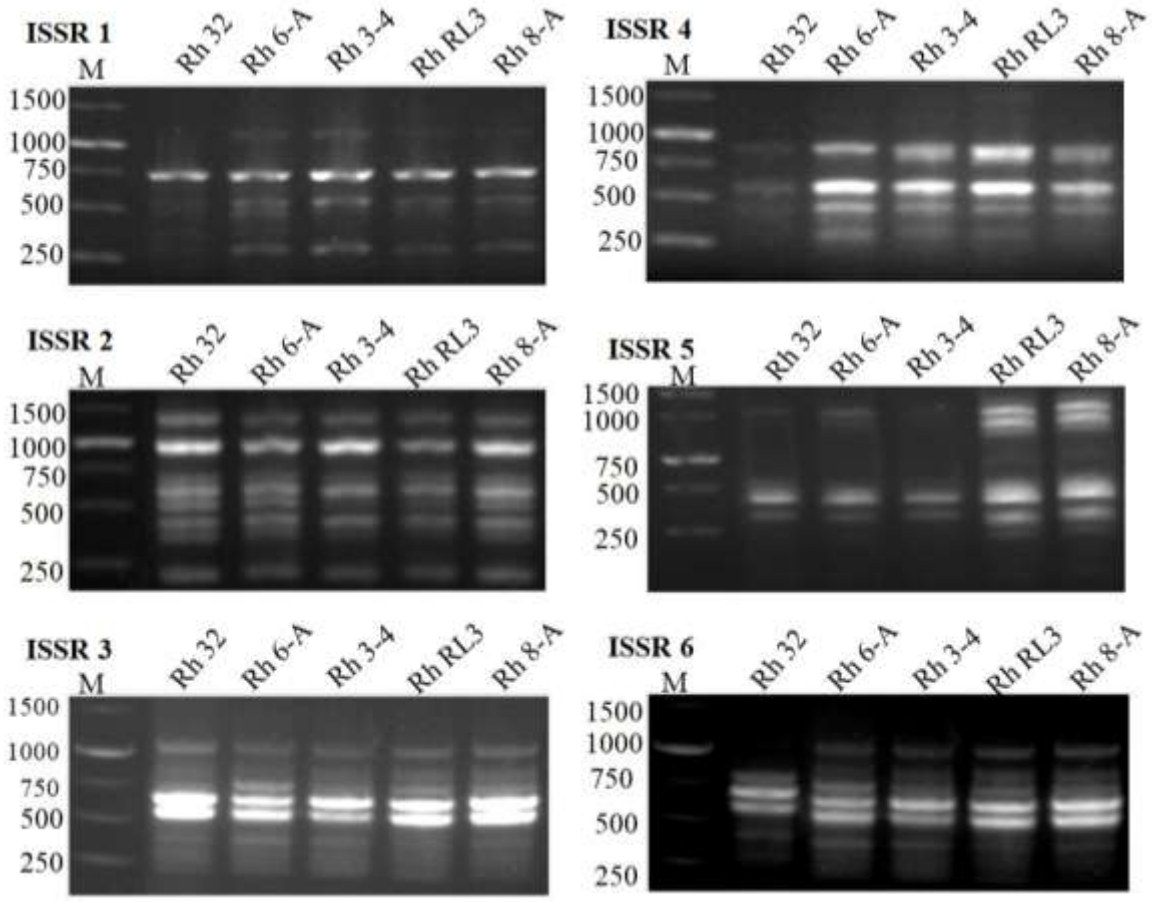

Fig. 1. Profiles of DNA amplification of the five rhizobial isolates (Agarose gel 1.5%, stained with ethidium bromide) revealed by six ISSR primers

Table 4. Similarity coefficients (Dice Measurement) of the five rhizobial isolates based on ISSR data analysis

Bacterial isolates	Rh 32	Rh 6-A	Rh 3-4	Rh RL3	Rh 8-A
Rh 32	1.00				
Rh 6-A	0.764	1.00			
Rh 3-4	0.745	0.931	1.00		
Rh RL3	0.764	0.871	0.828	1.00	
Rh 8-A	0.786	0.857	0.814	0.921	1.00

Table 5. Genetic distances, calculated as the total number of ISSR band differences among the five rhizobial isolates

Bacterial isolates	Rh 32	Rh 6-A	Rh 3-4	Rh RL3	Rh 8-A
Rh 32	0.00				
Rh 6-A	3.61	0.00			
Rh 3-4	3.61	2.00	0.00		
Rh RL3	3.61	2.83	3.16	0.00	
Rh 8-A	3.46	3.00	3.32	2.24	0.00

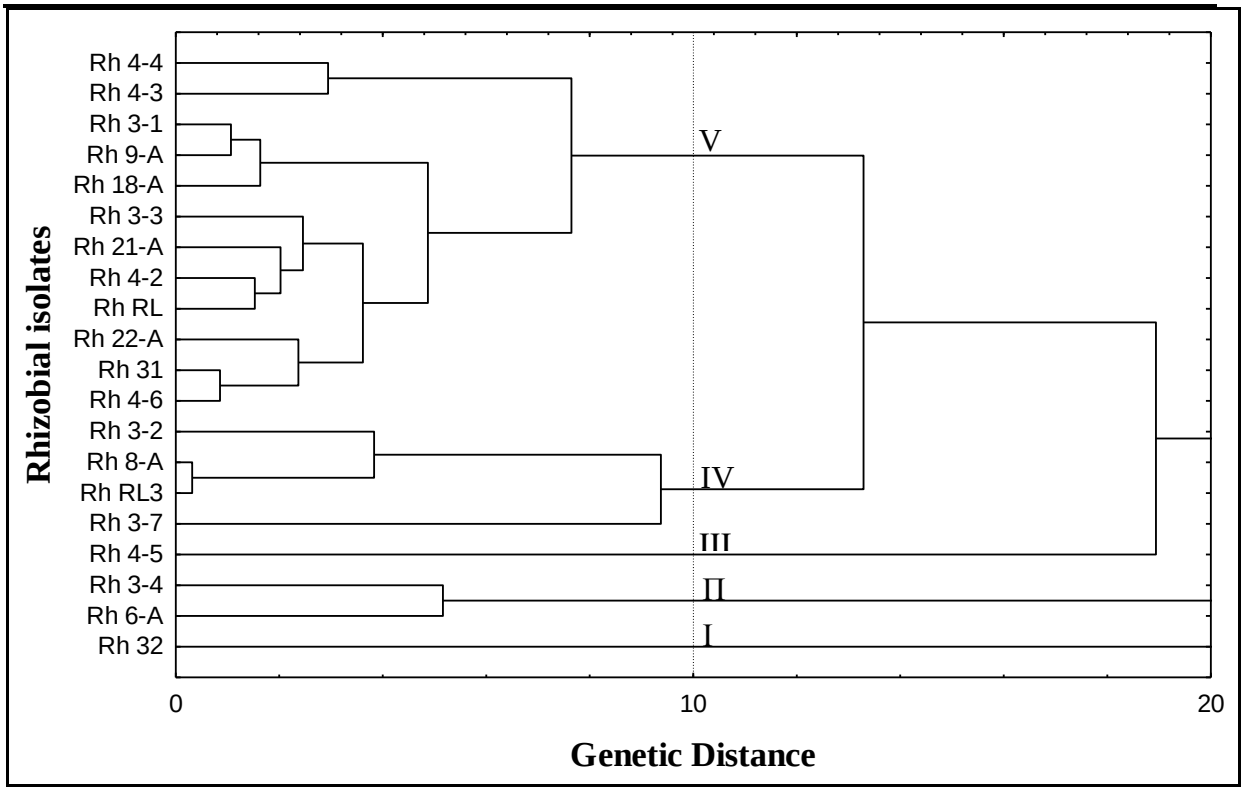

Fig. 2. Linkage dendrogram of the twenty rhizobial isolates based on morphological and physiological attributes

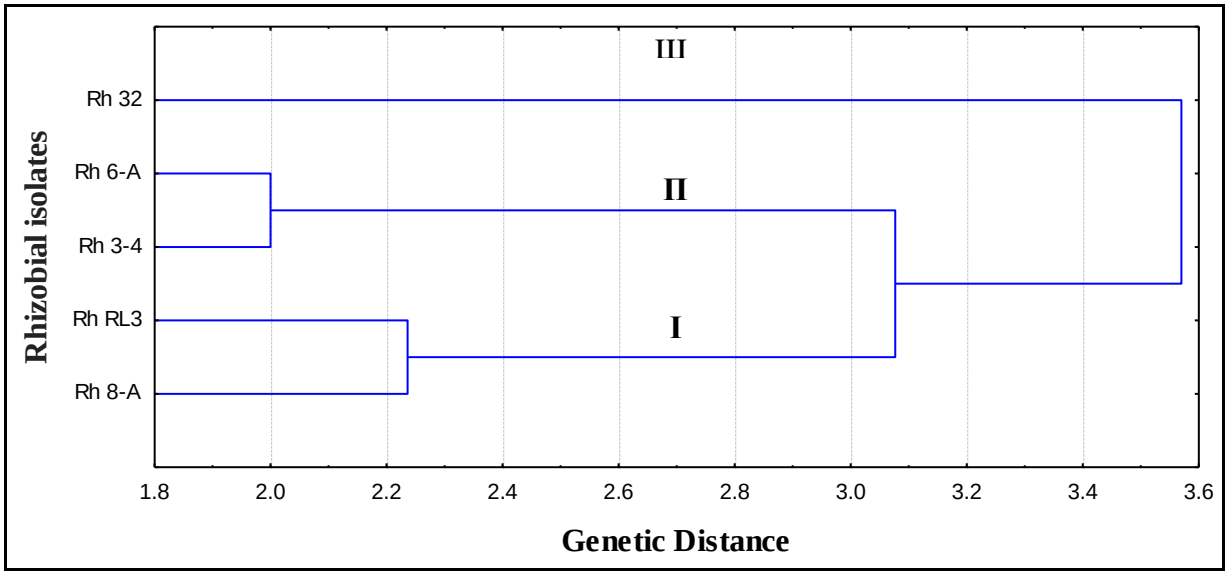

Fig. 3. Linkage dendrogram of the five promising rhizobial isolates based on their ISSR-PCR banding patterns

formed cluster I, Rh 6-A and Rh 3-4 isolates formed cluster П, and Rh 32 isolate formed the separated cluster ш. The results showed in Fig. 3 indicated that the five selected rhizobial isolates were falling close to each other. This might indicate that clustering analysis based on molecular data would be useful for selecting promising efficient rhizobial genotypes. These results add to the rapidly expanding of understanding the different genetic background of the studied rhizobial isolates.

DISCUSSION

Recently, there has been growing interest in using biofertilizers, which pushed us to isolate and characterize new nitrogen fixing isolates based on their own characteristics that qualify them to be remarkable nitrogen fixing bacterial strains. The selection of the five current rhizobial isolates was based on their ability to produce high biomass under complete and minimal media conditions, IAA production, and their efficiency to produce high nodule number/ plant. The significant variability among isolates, e.g. IAA production was discussed by **Palaniappan *et al.* (2010)**. The variation in nitrogen fixation efficiency of the rhizobial isolates under controlled conditions was consentient with that reported by **Arora *et al.*, (2018)** who investigated the efficiency of *Rhizobium* strains to nodulate the pigeon pea under pot conditions.

A primary concern of genetic characterization is to determine the genetic fingerprinting of a certain organism. The five rhizobial isolates that showed remarkable features out of the rest of the twenty rhizobial isolates were implemented to DNA fingerprinting using PCR-ISSR based method. DNA profiling methods use the extracted DNA samples to assess the variability of the microbial community and to discern specific patterns and correlations between DNA profile and a specific biological property. By using six ISSR primers, DNA fingerprinting profiles were generated as presented in figure 1. From which, it could be seen a correlation between the promising biological features of the five isolates and their DNA banding patterns. This would expand our knowledge about organism relationships and establishes the bases for traditional identification techniques (**Wang *et al.*, 2016**).

The phylogenetic and clustering analysis have emerged as powerful tools for grouping and selection of certain organisms. According to ISSR and morphological data, the clustering

analysis grouped the rhizobial isolates into separate clusters. The clustering pattern put the Rh 32 isolate into a separate cluster, which was isolated from Elmansoura city, and produced the highest quantity of IAA whether directly from biochemical test or indirectly from ISSR based analyses. This would suggest that this isolate has unique features and might be a possible candidate as nitrogen fixing inoculants. Interestingly, both analyses grouped both of Rh 6-A and Rh 3-4 isolates which were isolated from the same source (nodules) but collected from two different geographical locations in the same separate cluster. In the same context, both analyses grouped both of Rh 8-A and Rh RL3 isolates collected from the same location and the same source (rhizospheric) in the same separate cluster which visualize the close relationship between the physiological and molecular data. This may be due to the high conserved nature of ISSR regions, which support the using of ISSR molecular markers in the genotyping and profiling of rhizobial isolates. The phylogenetic analysis provides different evolutionary history for particular genes or organisms (**Krieg *et al.*, 2010; Drewnowska *et al.*, 2020; Huang *et al.*, 2020**).

The genetic distance values between the isolates were variable and not correlated with the geographical distribution. For instance, the lowest genetic distance values were obtained between Rh 6-A and Rh 3-4 isolates, While the highest genetic value was between Rh 32 isolate (Elmansoura city) and each of Rh 6-A isolates (Elmansoura city), Rh 3-4 isolate (Damietta city), and Rh RL3 isolate (Tallkha city), this may also explain the specificity of ISSR primers to amplify high conserved ISSR regions and interpret the possibility that such isolates have originated from the same genetic background of those isolates. Similar results were obtained by **Ismail *et al.* (2013)**, who reported that, the genetic homogeneity within and between rhizobial isolates are correlated to its genetics, not to the geographical locations in Egypt. These indicate the close genetic background between the rhizobial isolates.

Conclusion

The current work set out to investigate the genetic fingerprinting of the five selected rhizobial isolates from root nodules and rhizosphere of highly diversified agricultural areas in Egypt due to their remarkable features. The five isolates scored high biomass density, visualized efficient nodulation status, and produced high IAA quantity, which mean that they can act as plant growth promoters and efficient biofertilizers for legumes crops. These properties may qualify these rhizobial isolates to be used as a source of genes to improve the ability of other strains to accumulate nitrogen and promote plant growth. Molecular characterization and clustering analysis based on ISSR molecular markers and physiological properties would enable us to select more promising and efficient rhizobial isolates that can be used as sources for nitrogen-fixing inoculants. We concluded that the five rhizobial isolates will be promising as a source of genes for nitrogen fixation and plant growth-promotion.

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التنميط والتنوع الوراثى لبعض عزلات الرايزوبيوم الفعاله والمبشره مع نباتات الفول البلدى

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جمعت مجموعة من عشرين عزلة ريزوبيوم من الفول البلدي من محافظتين من جمهورية مصر العربية (الدقهلية ودمياط)، كما تم توصيف العزلات مورفولوجيا وفسيولوجيًا لفحص ودراسة نموها وأدائها التكافلي على نباتات الفول. وفقًا للاختبارات المعملية والحقلية، تم اختيار خمسة عزلات جذرية (Rh 32 ، Rh 6-A، Rh 3-4 ، Rh RL3 ، Rh 8-A) وإخضاعها لمزيد من التوصيف الجزيئي والكيميائي. تم إجراء التنميط الجيني لعزلات الريزوبيوم الخمس الواعدة باستخدام ستة بادئات ISSR. أنتج تضخيم الـ DNA البكتيري 37 موقعًا جينوميًا، من بينهم 54% Polymorphic و46% Monomorphic. تراوح معدل الـ Polymorphism بين 25% إلى 80% بمتوسط 54%. قسم تحليل المجموعات العنقودية البيانات المورفولوجية والفسيولوجية للعشرين عزلة من الريزوبيوم الى خمس مجموعات، وكانت عزلات الريزوبيوم الخمس المنتخبة تتقارب من بعضها البعض. قسم التحليل العنقودي بناء على بيانات الـ ISSR عزلات الريزوبيوم الخمس في أربع مجموعات. أظهر التحليل المعتمد على بيانات ISSR أن أقل مسافة وراثية كانت 2.00 بين عزلات Rh 6-A و Rh 3-4، بينما كانت أعلى مسافة وراثية 3.61 بين Rh 32 وكل من Rh 6-A و Rh 3-4 و Rh RL3. كان أعلى نسبة تشابه 0.931 بين عزلاتRh 6-A و Rh 3-4. بينما أقل تشابه كان 0.745 بين عزلات Rh 32 وRh 3-4. خلصنا إلى أنه يمكن استخدام تحليل المجموعات بناءً على البيانات الجزيئية في تسهيل انتخاب العزلات الريزوبيا التي ستكون واعدة كمصدر للجينات لتثبيت النيتروجين البيولوجي وتعزيز نمو النبات.

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