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Diversity of *Rhizobium leguminosarum* bv. *viceae* Strains Isolated from Different Schemes in Shendi Area

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A B S T R A C T

An attempt has been made to evaluate the diversity of sixteen *Rhizobium leguminosarum* bv. *viceae* strains isolated from different schemes in Shendi area. Morphological and cultural characteristics were performed viz: colony morphology, Gram staining and motility. Biochemical and physiological tests include acid - base production, oxidase and catalast tests were carried out. The effects of antibiotics on the growth of *Rhizobium* strains on YEMA media were tested using measurement of diameters of the growth inhibition zones. Growth of pure rhizobial isolates on (YEM) medium having variable range of pH (5.5 to 8.5) and different concentrations of NaCl (0.5 - 6.6 %) were recorded. The genetic diversity of the eleven studied strains were examined and screened for the polymorphism and were amplified using two different RAPD primers (OPC9 and OPY14). The isolates studied were motile, gram negative and rod shaped and catalase and oxidase positive bacteria. Regardless the location of isolation. *Rhizobium* strains tested showed significant differences ($P \leq 0.05$) in their sensitivity to the antibiotics. Strain $SHUOS_1F_{34}$, $SHUMAF_6$ and $SHUGF_{37}$ were the most sensitive strains where as strain $SHUJ_{15}F_2$, $SHUSSF_4$ and $SHUR_2F_{36}$ were the most tolerant ones to almost all antibiotics tested. With the exception of strain $SHUNOF_{35}$, which tolerate high pH values upto 8.5, the growth rate of all strains increased steadily with increasing pH from 5.5 reaching the maximum at pH 6.5 to 7.5 and declined then after to 8.5. In all the tested strains the growth rate decreased with increasing salt concentration from 0.5 upto 6%.The two RAPD primers gave amplification products and they were reproducible. A total of 88 fragments were detected representing 15 different loci with 100% polymorphism. The most relative lines were strains $SHUTR_2F_{36}$ and $SHUGO_3F_{42}$ with 79% similarity. According to the similarity indices, the eleven strains were grouped into four Clusters

Keywords: *Rhizobium leguminosarum, phenotypic characterization, genetic diversity, PCR.*

INTRODUCTION

Rhizobia are common group of small, rod-shaped, Gram-negative bacteria that collectively have the ability to produce nodules on the roots of leguminous plants. The taxonomy of rhizobia was based on the rate of growth of isolates on laboratory media and their selective interaction with their plant hosts. It was soon established that no strain could nodulate all plants, but that each could nodulate some legumes though not others (Long, 1989). This led to the concept of cross-inoculation groups, with organisms grouped according to the hosts they nodulated. Within the genus *Rhizobium* several strains nodulate a common host, but are distinct according to genetic and/or phenotypic properties and are therefore classified as distinct species (e.g. *R. tropic* and *R. etli*). However some strains cannot be distinguished other than by their host range, therefore the species is further classified into biovars (bv.) (e.g. *R. leguminosarum* is split into three biovars that nodulate, faba bean and peas) (Graham, , 1991).

Rhizobia in agricultural soil comprise a relatively small proportion of the total soil bacteria, varying from 0.1 to 8% . Difficulties arise when attempting to characterize the size, distribution and diversity of the rhizobial components of the soil micro- flora because the diversity of rhizobial strain residing in a particular soil can be considerable. These strains may not only differ in symbiotic capabilities, but also in other phenotypic and genetic characteristics (Brockwell , 1995).

In agricultural soil, the population diversity of a particular strains or species of rhizobia is enhanced by the presence of the cultivated legume it can nodulate . It follows that the overall diversity of soil rhizobia may be restricted to intra- specific variation where monoculture of cultivated legume species is practiced over a long time (Zahran, 2001).

The characterization of those populations has been performed using different strain typing methods depicting a considerable genetic heterogeneity between strains. Reliable identification of specific rhizobial strains is necessary for the study of their symbiotic association with plants. The characterization of new prospective isolates for application requires the development of easy, rapid and reliable methods for their identification (Selenska-Pobell , 1996). The size, shape, color and texture of colonies and the ability to alter the pH of the media are generally stable characteristics useful in defining strains of isolates. Tolerance to acid or alkali, , resistance to antibiotics ,and tolerance to salinity, were used by many researchers to determine a wide physiological diversity among tested isolates (Ahmad , 1984 and Maatallah , 2002). These phenotypic methods provide a valuable insight into rhizobial population structure and strain diversity. Thus, they are best regarded as preliminary screening methods for relative screening methods.

The previous methods used for distinguishing rhizobial strains were morphological, physiological and biochemical . However, these traditional methods of *Rhizobium* characterization frequently fail in the identification of strains within a species. So, it is necessary to obtain a better understanding of microbial diversity, molecular methods should be adopted for strain identification. Minimum standards for the description of new genera and species of *Rhizobium* are given by Graham (1991). Proper description and classification of the microorganisms should include numerical analysis of colonial and cultural characteristics and the analysis of representative strains by using measures from molecular studies that enable the identification of the microorganism. Methods for assessing strain diversity within species include the utilization of molecular techniques. Characterization of the *Rhizobium* genome at the molecular level is the most discriminating method for assesses the variability among strains and isolates of the bacteria (Thies , 2001). Molecular tools for the identification of bacteria are now available and are used routinely in laboratories.

Molecular techniques have helped to develop easy and quick methods to microbial characterization including works distinguish genera, species and even strains (Schneider and Brujin,1996; Giongo, , 2008). The polymerase chain reaction (PCR) and the use of primers corresponding to consensus repetitive sequences scattered in the Eubacteria genome can create highly characteristic patterns when distinguished in Agarose gels, providing well separation on strain level (Adiguzel, 2006).

Molecular techniques based on the polymerase chain reaction (PCR) are very convenient for characterization, because they are rapid, simple and discriminative. PCR has been found useful for rhizobial strain differentiation using GC-rich oligonucleotide primers. It has been possible to differentiate strains belonging to different colony morphology, (Muhammad, , 2008).

MATERIALS AND METHODS

Collection of samples

Sixteen *Rhizobium leguminosarum* bv. *viceae* strains were collected from faba bean plants randomly from different locations in Shendi area.These includes 6 strains from Gandato Agricultural Scheme (South Shendi) ,6 from Kaboshia Agricultural Scheme (North Shendi) and 4from Sayal Agricultural Scheme (west Shendi).

Morphological and cultural characteristics

Colony morphology

The morphological characteristics of the isolates were determined according to Lupwayi and Haque (1994). A loopful of rhizobial isolates from 48 hrs old broth culture was inoculated by streak plating onto YEM Agar and incubated at 28 ± 2^{o}C for 3-5days. After 5 days, colony diameter and morphology, colony texture were recorded as large mucoid large watery Elastic and buttery respectively.

Gram Staining and Motility test

The gram staining technique and motility test were done according to Benson (1994).

Biochemical and Physiological characteristics

The biochemical and Physiological tests were carried out in growth medium at 28^{o}C for 48hours incubation. All the tests were carried out with three replicates.

Acid-base production

To determine the ability of the rhizobial isolates to produce acid or alkaline in the medium, YEMA containing bromothymol blue (BTB) (0.025 w/v) was used. A loop full of the isolates from a 48 hrs old culture broth was streaked on to the YEMA BTB medium and incubated for 3-7 days so as to record the color changes of the medium (Jordan, 1984).

Oxidase test

A few drops of freshly prepared oxidase reagent was added on a piece of filtered paper in a clean Petri plate. Using a glass rod, a colony of the isolate was smeared on the filter paper and the paper was observed for any change in the colors of the reagent (Benson, 1994).

Catalase test

A thick growth of the test culture was immersed in 3ml of 3% H_2O_2 solution with the help of a sterile glass rod. Active bubbling within a few seconds showed a positive catalase activity (Benson, 1994).

Effects of Antibiotics on growth of Rhizobium

The effects of antibiotics on the growth of *Rhizobium* strains on YEMA media were tested as described by Bollich *et. al*. (1985) using measurement of diameters of the growth inhibition zones. The antibiotics used were Tetracycline 60ug/ml, Ciprofloxacin 1ug/ml, Chloromaphicol 30ug/ml, Streptomycin 40ug/ml, Nalidixic acid 30ug/ml, and Erythromycin 40ug/ml.

Three replicates were made per treatment. The diameters of the zones of inhibition were measured after 3 and 5 days of incubation for *rhizobium* strains. The experiment was arranged by using completely Randomized Design (CRD). Comparison between the treatments were done by two way analysis of variance (ANOVA) the means were separated by least Significance Difference test (LSD), ($P \leq 0.05$)**.**

Effects of pH on growth of Rhizobium

The capacity of each rhizobial isolate to grow on acidic and alkaline media was determined by inoculating each isolate on YEMA adjusted at a pH of 5.5 , 6 , 6.5 , 7 , 7.5 ,8 , 8.5 using NaOH and HCl as described by Bernal and Graham (2001) all the plates were incubated at 28°C for 72 hours and YEMA medium plates were used as controls.

Effects of Salt on growth of Rhizobium

The ability of the isolates to grow at different level of salt concentrations was determined by inoculating each isolate on the YEMA media containing .05% , 1% , 2% , 3% , 4% , 5% , 6% of NaCl as indicated in Bernal and Graham(2001).

Molecular Characterization of Rhizobium

Genomic DNA Extraction

DNA extraction from isolates was done according to the boiling centrifugation method reported by Miller ,(1999). A single colony was grown over night at 28 °C in YEM broth. Bacterial cell were precipitated by a centrifugation at 13000 rpm for 10 minute in a micro-centrifuge (Sanyo). The supernatant was discarded and the pellets were resuspended in 500ml deionized water. The suspension was boiled for 10 minutes in a water bath and then immediately cooled on ice. Extracted DNA was then stored refrigerated until used.

Agarose gel electrophoresis of the extracted DNA

The extracted DNA was electrophoresed in 1.5% agarose gel [0.75g agarose dissolved in 50 ml of 1x TBE buffer (0.089 mol/L Tris-borate, and 0.002mM EDTA, pH 8.00)] (Sawada , 1995). Then 2 µl of ethidium bromide (10 mg/ml) were added Prior to casting the gel, the comb was adjusted and the gel was poured (making sure that there were no bubbles). While the gel was solidifying, DNA mixtures were prepared for electrophoresis as follows: 1 µl of each DNA sample was transferred to a clean Eppendorf tube and 3µl of loading dye (bromo phenol blue dye) was added to the DNA sample. The content was mixed several times using a micropipette. The comb was removed with gentle back and forth motion and the gel was then immersed in 1x TBE buffer. The buffer was added until it reached a level approximately 3-5 mm above the gel surface. The sample mixtures were loaded into the wells using plastic-tipped micropipettes. 1Kb ladder (Invitrogen) was used as a molecular weight marker. The apparatus (Habaib, U.K, 9H 310083) was closed and the power was turned on, the voltage was adjusted to 75V (400mA) and the running was continued without cooling for 20 minutes after which the gel was visualized under trans illumination

cabinet (Model TM-10E, Uvitec. Product) and image was captured and photographed. Extracted DNA was then stored refrigerated until used as a template for PCR amplification.

Polymerase chain reaction (PCR)

For genetic diversity studies two RAPD primers were used to amplify the genomic DNA. The primers were purchased from Gene link, Inc. and Operon Tech., NY 10532. These were 10 oligonucleotide OPC9 primer (CTCACCGTCC), and OPY14 (GGTCGATCTG).

PCR amplification reactions were carried out in a total volume of 20 µl. Each PCR mixtures contained (Final concentration): 5X FIRE Pol PCR Master Mix (Ready to load), 5 X reaction buffer (0.4 M Tris-HCL, 0.1 M (NH_4) SO_4, 0.1% w/v Tween 20), 12.5 Mm dNTPs, 50 ng of the primer under test, 1 U Taq polymerase and 20 mg template DNA.

The amplification program used consisted of one cycle at 94°C for 5min, followed by 35 cycles of initial denaturation at 94°C for 1min, annealing at 32°C for 3min, extension at 72°C for 2 min and a final extension step at 72°C for 10 min.

RESULTS AND DISCUSSION

Morphological, cultural and Biochemical characteristics

The isolates studied were motile, gram negative and rod shaped bacteria as revealed by Grams staining technique and motility test. The isolates changed the YEMA-BTB medium to yellow color and did not absorb Congo red on YEMA-CR media. the coloration of colonies was milky-white translucent with a circular shape, with regular borders, shiny and raised, showing intermediate to high production of mucus, after 3 to 5 day of growth on YEM A medium at 28^{o}C. Furthermore, all of the isolates colony diameter ranging from 2 to 5 mm (Table 1).

Based on colony size, texture and confluent growth rate with gum production, the isolates were categorized as fast growing rhizobia. They also changed the YEMA-BTB medium to yellow color indicating that the isolates were acid producing root nodule bacteria.. All these features are characteristics of fast growing rizobia including the cross – inoculation group of Rhizobium legumionosarum bv.viciae that nodulates member of the tribe viceae (Faba bean, pea). (Somasegaran and Hoben, 1994). These results confirmed those obtained by Singh , (2008) .

Microscopic examination revealed that all the isolates were catalase and oxidase positive as confirmed by the liberation of effervescence of oxygen around the bacterial colonies and change in color of the oxidase strip, respectively. The fact that Rhizobium leguminosarum bv. viciae were calalase and oxidase positive was stated by Lupwayi and Hague, (1994). Morphological and physiological characterizations. Reliable identification of specific rhizobial strains is necessary for the study of their symbiotic association with plants. From the root nodules of lentil (Lens culinaris Medic) Selenska-Pobell , (1996) found that 12 rhizobial isolates were obtained. Fast growing rhizobial strains showed sufficient growth and more gum production having a mean generation time of 24-36 h., while slow growing rhizobial strains showed sufficient growth but less gum production, having a mean generation time of 36-48 h. The colonies obtained were gummy, translucent and circular with entire or smooth margins. All the strains were gram negative and rod shaped as revealed by Gram's staining technique.

Effects of antibiotics on the diameters of inhibition zones of Rhizobium strains

As shown in Table (2), the antibiotics used in the study significantly ($P \leq 0.05$) affected the growth of *Rhizobium* strains. Ciprofloxacin antibiotic formed a wider inhibition zone of growth showing a higher sensitivity of the rhizobial strains tested, followed by Streptomycen and Tetracycline respectively. No significant differences were found between Nalidixic acid, Erythromycin and Chloromaphicol antibiotics. where the rhizobial strains showed a high tolerance to them.

The location from where the isolates were isolated have no significant differences regarding the tolerance of the strains although strains isolated from Kaboshia Agricultural Scheme were more tolerant than those from other locations.

Regardless the location of isolation, *Rhizobium* strains tested showed significant differences ($P \leq 0.05$) in their sensitivity to the antibiotics. Strain $SHUOS_1F_{34}$, $SHUMAF_6$ and $SHUGF_{37}$ were the most sensitive strains where as strain $SHUJ_{15}F_2$, $SHUSSF_4$ and $SHUR_2F_{36}$ were the most tolerant ones to almost all antibiotics tested.

The antibiotic-strain interaction showed a significant differences ($p \leq 0.05$) with no specific correlation between the antibiotic and the strain.

The pattern of antibiotic resistance is generally similar to the findings of the same antibiotics on isolates of *Rhizobium leguminosarum* bv. *viceae*. Some of these antibiotics (Streptomycin and Erythromycin) were also used to define the different clusters of faba bean *Rhizobiam* isolated from the USA (Brockman and Bezdicek, 1989). Antibiotic resistance was also reported to be useful in the preliminary screening of taxonomic groups of other root nodule bacteria (Shishido and Pepper, 1990; Chen, 2002).

The wide range of antibiotic tolerance partially explains the success under field conditions of the inoculant strains currently recommended, as this tolerance is a possible mechanism to overcome antagonism exerted by other organisms in the soil. Some studies show a correlation between the growth rate and rhizobia tolerance to some antibiotics. (Odee , 1997) where as other studies found no correlation between rhizobia growth rate and antibiotic tolerance(Maâtallah , 2002).

Effects of pH on the growth of the strains.

With the exception of strain $SHUNOF_{35}$, which tolerate high pH values upto 8.5, the growth rate of all strains increased steadily with increasing pH from 5.5 reaching the maximum at pH 6.5 to 7.5 and declined then after to 8.5. (Table 3). Two strains from Gandato Agricultural The strains scheme viz: $SHUOS_4F_{49}$ and $SHUGL_2F_3$ and two strains from Kaboshia Agricultural scheme viz: $SHUSB_2F_{22}$ and $SHUOMF_{32}$ with strain shunof35 from Sayal Agricultural scheme tolerated all the pH tested showing slight growth rate at low or high pH values with abundant growth at pH range 6.5 - 7.5. The strains differ widely in their tolerance to the acidic or alkaline medium as some of them (Table 3).

PH tolerance Rhizobia appear to be varying in their symbiotic efficiency under acidic and alkaline conditions. Ali , (2009) reported that all the tested isolates showed very poor growth at pH 4.0. There was considerable increase in the growth rate with increasing pH upto 7.0. Somewhat similar to this, Rodrigues , (2006) quoted that the pH 6.5-7.0 is the most optimum pH for the growth of root nodulating bacteria. However, inhibitory effect of elevated pH (above 7.0) was clearly visible on the growth response of rhizobia since moderate growth was recorded for majority of the isolates which similar to the finding of this study.

Effects of salt concentration on the growth of the strains

All the tested strains tolerated salt concentrations of 0.5 and 1% showing abundant growth except strain $SHUR_1F_{45}$. At salt concentration of 2%. the strains vary in their tolerance to the salt between abundant and slight growth, where as all of the strains tested grow slightly at 3% salt concentration. Increasing salt concentration resulted in decreasing the growth depending on the strain.

In all the tested strains the growth rate decreased with increasing salt concentration from 0.5 upto 6%. Half of the strains grow normally in all salt concentrations giving abundant growth upto salt concentration of 2% and decreased upto 6% salt concentration. No clear differences were observed between the locations of strain isolation.

Tolerance to NaCl stress is a very complex phenotype that involves not only the bacterial ability to tolerate the stress but also the swiftness to respond and adapt to the environmental change. Decreased growth of rhizobial isolates with increasing salt concentration was registered by Ali , (2009). Similar to this, Thrall , (2008) were of the view that increasing salt concentrations may have a detrimental effect on rhizobial populations as a result of direct toxicity as well as through osmotic stress.

Genetic diversity of Rhizobium strains

The genetic diversity of the eleven studied strains were examined and screened for the polymorphism between the them**.** The strains were amplified using two different RAPD primers (OPC9 and OPY14), The two RAPD primers gave amplification products and they were reproducible.

A total of 88 fragments were detected for the eleven strains representing 15 different loci with 100% polymorphism (Fig 1, 2). Strain $SHUMAF_6$ gave no amplification product with primer OPY14. Molecular techniques based on the polymerase chain reaction (PCR) has been used to differentiate strains belonging to different colony morphology variants of the strain USDA110, which differ in symbiotic nitrogen fixation (Paffetti , 1996). Random amplified polymorphic DNA (RAPD) was also used to assess the genetic diversity of rhizobial strains from various districts of Punjab, Pakistan.

Genetic Similarity Analysis

The dendrogram tree among the studied *Rhizobium* isolates based on RAPD marker were calculated using Jaccard's coefficient depending on the similarity matrix present in Fig (1). The analysis was based on the number of markers that were similar between any given pair of isolates. The most relative lines were strains $SHUTR_2F_{36}$ and $SHUGO_3F_{42}$ with 79% similarity; while the most distant were strains $SHUMAF_{39}$ and $SHUMAF_6$ as well as $SHUSSF_4$ and $SHUMAF_6$ with 0.00 % similarity percentage, (Table 5). The polymorphism percentage was lower than that obtained by Hameed (2004) in Rhizobium, Bradyrhizobium and Agrobacterium strains (99.8%).

According to the similarity indices, the eleven strains were grouped into four Clusters. Cluster 1 contains strains $SHUWDF_{29}$, and $SHUNOF_{35}$, Cluster 2 contains strains $SHUMAF_6$, and $SHUOS_1F_{34}$, Cluster 3 contains strains $SHUMAF_{39}$, $SHUSSF_4$, and $SHUTOF_{44}$, Cluster 4 contains strains $SHUGO_3F_{42}$, $SHUTR_2F_{36}$, $SHUKB_1F_8$, and $SHUJZ_{15}F_2$ (Fig 3).

Table 1. Morphological and Biochemical Characteristics of *Rhizobium* Strain

Source	Strain	Antibiotics					
		T	Cf	C	S	Na	E
Gandato	$SHUJZ_15F_2$	0.001	0.001	0.001	7.334	0.001	0.001
	$SHUOS_1F_{34}$	15.667	22.667	10.667	20.333	15.000	10.000
	$SHUTOF_{44}$	17.333	12.000	6.667	12.667	6.334	3
	$SHUOS_4F_{49}$	14.333	0.001	7.334	20.667	10.333	10.667
	$SHUWDF_{29}$	9.667	5.001	8.334	7.001	3.333	3.001
	$SHUGL_2F_3$	25.667	16.667	0.001	9.667	6.334	3.001
Kaboshia	$SHUTR_2F_{36}$	0.001	0.001	0.001	8.667	3.667	8.667
	$SHUMAF_{39}$	7.334	12.000	3.334	9.000	0.001	0.001
	$SHUTR_1F_{45}$	8.334	13.334	3.334	15.667	0.001	6.667
	$SHUKB_1F_8$	28.333	19.667	10.000	18.667	9.000	8.334
	$SHUOMF_{32}$	12.667	18.667	9.000	12.667	11.000	9.667
	$SHUGO_3F_{42}$	10.667	15.667	0.001	11.667	0.001	0.001
Sayal	$SHUSSF_4$	0.001	3.334	0.001	6.334	0.001	0.001
	$SHUMAF_6$	17.333	20.000	11.667	11.667	13.333	12.333
	$SHUSKF_{16}$	10.000	19.000	9.667	13.000	10.667	6.334
	$SHUNOF_{35}$	13.000	15.000	9.000	6.000	9.667	6.000

Table 2. Effect of antibiotics on the diameters of inhibition zones of *Rhizobium* strains (mm)

Source	Strains	Oxidase	Catalase	Motility	Color in Congo red	Bromo thymol medium	with colony color	Mucocity	Colony shape	Cell shape	Gram stain reaction
Gandato	$SHUJZ_15F_2$	+	+	+	White	Yellow		+	Circular	Rod	-Ve
	$SHUOS_1F_{34}$	+	+	+	White	Yellow		+	Circular	Rod	-Ve
	$SHUTOF_{44}$	+	+	+	White	Yellow		+	Circular	Rod	-Ve
	$SHUOS_4F_{49}$	+	+	+	White	Yellow		+	Circular	Rod	-Ve
	$SHUWDF_{29}$	+	+	+	White	Yellow		+	Circular	Rod	-Ve
	$SHUGL_2F_3$	+	+	+	White	Yellow		+	Circular	Rod	-Ve
Kboshia	$SHUTR_2F_{36}$	+	+	+	White	Yellow		+	Circular	Rod	-Ve
	$SHUMAF_{39}$	+	+	+	White	Yellow		+	Circular	Rod	-Ve
	$SHUTR_1F_{45}$	+	+	+	White	Yellow		+	Circular	Rod	-Ve
	$SHUKB_1F_8$	+	+	+	White	Yellow		+	Circular	Rod	-Ve
	$SHUOMF_{32}$	+	+	+	White	Yellow		+	Circular	Rod	-Ve
	$SHUGO_3F_{42}$	+	+	+	White	Yellow		+	Circular	Rod	-Ve
Saval	$SHUSSF_4$	+	+	+	White	Yellow		+	Circular	Rod	-Ve
	$SHUMAF_6$	+	+	+	White	Yellow		+	Circular	Rod	-Ve
	$SHUSKF_{16}$	+	+	+	White	Yellow		+	Circular	Rod	-Ve
	$SHUNOF_{35}$	+	+	+	White	Yellow		+	Circular	Rod	-Ve

SD for *Rhizobium* = 2.430
LSD for Antibiotics = 1.241
LSD for *Rhizobium**Antibiotics = 5.952
(T = Tetracycline; Cf = Ciprofloxacin; C = Chloromaphicol; S = Streptomycin; Na = Nalidixic acid; E = Erythromycin)

Table 3. Effect of pH level on the growth of *Rhizobium leguminosarum* bv *viceae* strains

Source		pH Concentrations						
	Strains	5.5	6	6.5	7	7.5	8	8.5
Gandato	$SHUJZ_1 5F_2$	+	+	++	++	++	+	_
	$SHUOS_1F_{34}$	_	+	+	+	+	+	_
	$SHUTOF_{44}$	_	+	+	+	+	_	_
	$SHUOS_4F_{49}$	+	+	++	++	++	+	+
	$SHUWDF_{29}$	+	+	++	++	++	++	+
	$SHUGL_2F_3$	+	+	+	++	++	+	_
Kaboshia	$SHUR_2F_{36}$	+	+	++	++	++	+	_
	$SHUMAF_{39}$	_	+	+	+	+	+	_
	$SHUTR_1F_{45}$	_	+	+	+	+	_	_
	$SHUKB_1F_8$	+	+	++	++	++	+	+
	$SHUOMF_{32}$	+	+	++	++	++	++	+
	$SHUGO_3F_{42}$	+	+	+	++	++	+	_
Sayal	$SHUSSF_4$	+	+	+	++	++	+	_
	$SHUMAF_6$	_	+	+	+	+	_	_
	$SHUSKF_{16}$	_	_	+	++	++	_	_
	$SHUNOF_{35}$	+	+	+	++	++	++	++

No growth ; + Slight growth; ++ abundant growth

Table 4. Effect of salt concentration on the growth of *Rhizobium leguminosarum* bv. *viceae* strains.

Source		Salt Concentrations						
	Strains	0. 5%	1%	2%	3%	4%	5%	6%
Gandato	$SHUJZ_1 5F_2$	++	++	++	+	+	+	+
	$SHUOS_1F_{34}$	++	++	+	+	_	_	_
	$SHUTOF_{44}$	++	++	++	+	_	_	_
	$SHUOS_4F_{49}$	++	++	+	+	_	_	_
	$SHUWDF_{29}$	++	++	+	+	+	+	_
	$SHUGL_2F_{31}$	++	++	++	+	+	_	_
Kaboshia	$SHUR_2F_{36}$	++	++	++	+	+	+	+
	$SHUMAF_{39}$	++	++	++	+	+	+	+
	$SHUTR_1F_{45}$	+	+	+	+	+	_	_
	$SHUKB_1F_8$	++	++	++	+	+	+	+
	$SHUOMF_{32}$	++	++	+	+	+	+	+
	$SHUGO_3F_{42}$	++	++	++	+	+	+	+
Sayal	$SHUSSF_4$	++	++	++	+	+	+	+
	$SHUMAF_6$	++	+	+	+	+	+	_
	$SHUSKF_{16}$	++	++	++	+	+	+	+
	$SHUNOF_{35}$	++	++	++	+	+	+	_

- No growth ; + Slight growth; ++ abundant growth

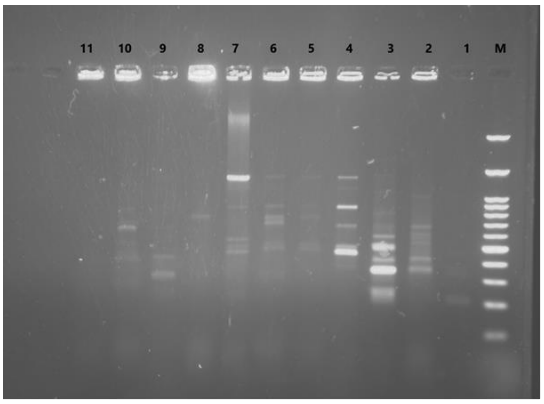

Figure 1. RAPD- PCR amplification of DNA isolated from *Rhizobium* isolates using primer OPC9. (1= $SHUWDF_{29}$; 2= $SHUTOF_{44}$; 3= $SHUOS_1F_{34}$; 4=$SHUSSF_4$; 5= $SHUTR_2F_{36}$; 6= $SHUJZ_{15}F_2$; 7= $SHUMAF_{39}$; 8= $SHUKB_1F_8$; 9=$SHUGO_3F_{42}$; 10= $SHUMAF_6$; 11= $SHUNOF_{35}$)

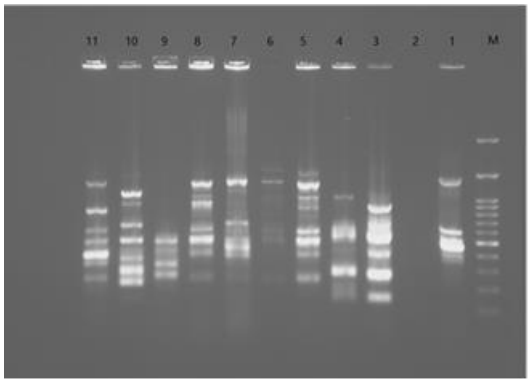

Figure 2. RAPD- PCR amplification of DNA isolated from *Rhizobium* isolates using primer OPY14 . (1= $SHUWDF_{29}$; 2= $SHUTOF_{44}$; 3= $SHUOS_1F_{34}$; 4= $SHUSSF_4$; 5= $SHUTR_2F_{36}$; 6= $SHUJZ_{15}F_2$; 7= $SHUMAF_{39}$; 8= $SHUKB_1F_8$; 9=$SHUGO_3F_{42}$; 10= $SHUMAF_6$; 11= $SHUNOF_{35}$)

Table 5. Similarity matrix between different tested strains of *Rhizobium* constructed from RAPD- PCR banding pattern

Strains	$SHUNOF_{35}$	$SHUMAF_6$	$SHUGO_3F_{42}$	$SHUKB_1F_8$	$SHUMAF_{39}$	$SHUJZ_{15}F_2$	$SHUTR_2F_{36}$	$SHUSSF_4$	$SHUOS1F_{34}$	$SHUTOF_{44}$	$SHUWDF_{29}$
$SHUNOF_{35}$	1.00										
$SHUMAF_6$	0.13	1.00									
$SHUGO_3F_{42}$	0.36	0.23	1.00								
$SHUKB_1F_8$	0.31	0.17	0.71	1.00							
$SHUMAF_{39}$	0.36	0.00	0.47	0.54	1.00						
$SHUJZ_{15}F_2$	0.36	0.20	0.47	0.67	0.64	1.00					
$SHUTR_2F_{36}$	0.38	0.25	0.79	0.77	0.50	0.62	1.00				
$SHUSSF_4$	0.33	0.00	0.46	0.42	0.67	0.36	0.38	1.00			
$SHUOS_1F_{34}$	0.50	0.50	0.46	0.31	0.15	0.25	0.50	0.20	1.00		
$SHUTOF_{44}$	0.45	0.08	0.64	0.40	0.58	0.36	0.57	0.60	0.33	1.00	
$SHUWDF_{29}$	0.50	0.20	0.23	0.27	0.20	0.20	0.25	0.29	0.50	0.18	1.00

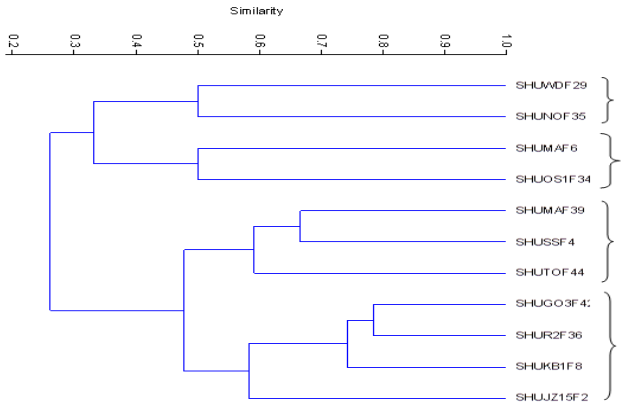

Figure 3. Dendrogram of *Rhizzobium* isolated from Faba bean *(Vicia faba* L.) based on RAPD-PCR

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