Detection of Genetic Variation Among Three Isolates of *R. leguminosarum* Using Protein, Isozyme and DNA Fingerprints

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Abstract: Molecular markers, protein and DNA fingerprints were used to analyze the genetic variations among three *Rhizobium leguminosarum*. The frequency of genetic variability was detected in three isolates of *R. leguminosarum*. Variation in protein contents (89.25, 92.27 and 90.75 mg/g), protein banding pattern (10, 12 and 11 polypeptide bands) was observed for the three isolates of *R. leguminosarum* M, IS and F, respectively, with 40% polymorphic bands. The variation in peroxidase isozymes was 4, 6 and 5 bands, respectively, with polymorphic bands of 37.5%. Variation in each of protein banding pattern and peroxidase isozymes among the three isolates suggest that DNA fingerprint analysis could be used to show rapid and precise information about genetic variability. Ten arbitrary base primers were successfully used to amplify DNA fragments from three isolates. Three arbitrary 10 base primers (OP07, OP12 and OP18) revealed characteristic fragments where as 20% polymorphic fragments related to total amplified DNA fragments. Monomorphic 48% common amplified fragments as well as unique (genetic markers) 32% for the three *R. leguminosarum* isolates. The frequency of genetic variability (polypeptide, isozymes and DNA) was detected among 3 isolates of *R. leguminosarum* dependent somaclonal variation.
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1. Introduction

Modern developments is molecular biology have exploited the use of polypeptide fractions, isozymes and DNA fingerprints for genetic variability detection and represent on active area of research in microorganisms (Williamson, 1991). In Egypt, species of *Rhizobium* spp. are generally regarded as biofertilizers for legume crops; they attack causing considerable increasing of yield and quality of the produce. The impact of the *Rhizobia* on a crop and the relationship between the *Rhizobium* and host differ between *Rhizobium* species.

Most of published studies on detection of genetic variability and gene mutations were concentrated in the variations in chromosomes (genomes), isozyme polymorphism and biochemical diversity. A single set of arbitrary sequence 10 mers may be used for fingerprinting any species. The many advantages of RAPD markers over RFLPs or isozymes and protein markers accelerated the adoption of RAPD technology for the construction of genetic maps and fingerprinting (Tingey and Del Tuto, 1993). Wöstemeyer and Kreibich (2002); Sharma (2003); Swelim (2005); Aiat (2006) and Shash (2008) recoded that, the quality of DNA markers as RAPD PCR in detection genetic variability is well established for many microorganism.

Therefore, this study was conducted to employ SDS-PAG, DISC-PAGE and PAPD-PCR analyses as simple tools to use the molecular markers for the detection of genetic variability among *R. leguminosarum* isolates. Such marker(s) could also be used to assess the genetic relationships of three isolates *R. leguminosarum*.

2. Material and Methods

2.1. *Rhizobium leguminosarum* isolates

Three *R. leguminosarum* isolates namely M, IS and F were grown on yeast extract mannitol broth medium (YEM). These isolates were selected from fourty root nodules of faba bean plants from different locations (Amer, 2008a; b).

2.2. Determination of protein

Protein content was determined according to Bradford (1976) using bovine serum albumin as a standard.

2.2.1 Protein analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as

described by Laemmli (1970) under reducing condition in discontinuous electrode buffer system. Protein samples were denatured and reduced completely by mixing the protein with in equal vol. of 2X sample buffer (Lucius *et al*, 1987) and the mixture was heated in a water bath at 92°C for 5 min and chilled on ice buffer. Treated proteins were centrifuged at 12000 rpm for 10 min. Electrophoresis was carried out at room temperature at a constant current at 25 mA for 1 hr. followed by 30 mA for 4 hrs. At the end of run, the gel was stained with Coomassie Brilliant Blue R-250 and destained in the stain solvent then photographed.

2.3. Peroxidase (PRX) isozymes electrophoresis

Electrophoresis of isozymes was performed according to Stegemann *et al.* (1986) and applied for three isolates of *R. leguminosarum* using PRX enzyme staining system. Peroxidase revealed the most variables with good-determined banding patterns.

2.4. Isolation of genomic DNA

Extraction of total DNA was performed according to the method of Wulff *et al.* (2002).

2.5. DNA amplification

Random amplified polymorphic DNA (RAPD) analysis was applied according to Williams *et al.* (1990) using 10 mers of five random oligonucleotide primers obtained from Metabion International AG. Lena-Christ-Str., Martinsried Deutschland as shown in table (1). Amplification was performed in 30 µl of reaction mixture containing 2.0 µl template DNA (25 mg, 2.0 µl *Taq* DNA polymerase (unit), 3.0 µl DNTPs (25 mol of each dATP, dCTP, dGTP and dTTP), 3.0 µl $MgCl_2$ (25mM), 3.0 µl PCR buffer (10X), 2.0 µl random primer (10 p mole) (Table 1) and 15.8 µl deionized water. The mixture was assembled on ice, overlaid with a drop of mineral oil. The amplification was carried out in DNA thermal cycle (MWG-Biotech Primuse) programmed as follows: Once cycle at 94°C for 4 min, and then 40 cycles; each cycle at 94°C for 30 sec (denaturation); 35°C for 1 min (annealing) and 72°C for 2 min (extension). One cycle at 72°C for 2 min then 4°C.

2.6. Gel electrophoresis analysis

All electrophoresis were carried out using a Pharmacia (GN-100) submorine gel electrophoresis apparatus. Agarose gel 1% was prepared in 1X TBE buffer (90 mM Tris-borate, 2 mM EDTA, pH 8.3). Total sample volume of 10 µl (5 µl of RAPD-PCR product, 4 µl dH_2O and 1 µl 6X loading dye) of each was loaded in gel wells and electorophoressed for 1.5 hr. The gel was stained with ethidium bromide solution (0.5 µg/ml) for around 10-15 min. DNA was visualized on a UV transillminator (1-254 nm) and photographed.

Table 1. Oligonucleotide sequences of the random primers.

Infinitive primer	Nucleotide sequences 5' → 3'
OPB-05	TGCGCCCTTC
OPB-07	GGTGACGCAG
OPB-10	CTGCTGGGAC
OPB-12	CCTTGACGCA
OPB-18	CCACAGCAGT
OPT-20	GACCAATGCC

3. Results

Variability among three *R. leguminosarum* in somaclonal variation should independent of bacterial genetics under environment to stress. Protein, isozymes and DNA were differed quantity and quality. Whereas, amino acid sequence of polypeptides are dependent on nucleotide sequence of their coding genes. Protein, isozymes and genomic DNA analysis detect the genetic variability among isolates of *R. leguminosarum*.

3.1. Protein banding patterns

The Results recorded in table (2) showed the differences in protein content, protein fractions and density, whereas, IS isolate showed to have the highest value of protein content followed by F isolate and M isolate of *R. leguminosarum*. SDS-PAGE profile of protein extracted from the three *R. leguminosarum* isolates was presented in table (2) and Fig. (1). It showed a total of 33 bands with different molecular weight values ranged from 200 to 8 KDa.

The protein bands of the three isolates varied in number and density of bands, whereas, M, IS and F isolates showed a total 10, 12 and 11 protein bands, respectively. The electrophoretic banding pattern of three *R. leguminosarum* isolates showed either absence or presence of different polypeptides in these three isolates. Six bands were polymorphic. They had the molecular weights of 150, 100, 89, 70, 40 and 8 KDa. Other bands were for only one isolate, such as 60, 30 and 12 KDa for F, IS and M isolates, respectively. Some of these bands appeared in each of the three isolates such as 200, 120, 50, 25, 20 and 15 KDa which were considered monomorphic ones. The presence of variability (polymorphic) among *R. leguminosarum* isolates was 40% in relation to the total number of bands.

3.2. Peroxidase iszoymes

Results of peroxidase isozymes are shown in table (3) and Fig. (1). The total number of peroxidase iszoymes shown in all three *R. leguminosarum* isolates were 8 isozyme bands. The number of these isozyme bands were 4, 6 and 5 for the isolates M, IS and F, respectively. The variability analysis of three isolates displayed a total of 8 bands. Of the eight isozyme bands, 3 bands of them were polymorphic, 2 others were common bands (monomorphic) over three isolates and 3 were unique bands. The percent of variability among 3 *R. leguminosarum* isolates was 37.5% relation to total peroxidase iszoymes.

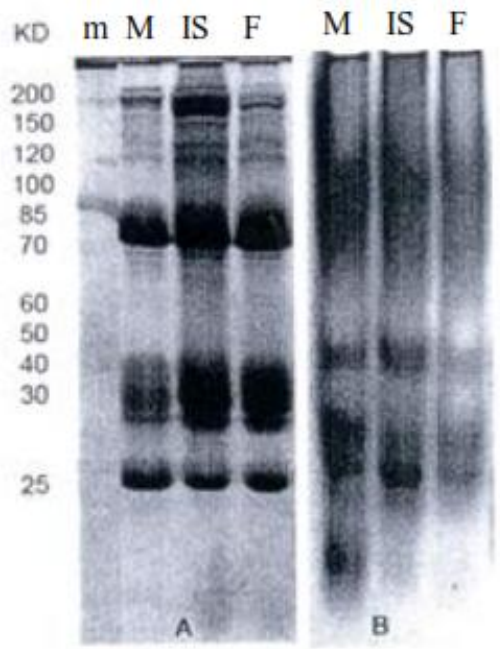

Figure 1. Illustrate the protein analysis and isozymes,

A. SDS-PAGE 12% of protein patterns extracted from 3- *Rhizobium* isolates.

B. DISC-PAGE 14% of polypeptides peroxidase isozymes of 3- *Rhizobium* isolates.

m = Marker.

M, IS, F = *Rhizobium* isolates.

3.3. RAPD-PCR analysis

The genetic diversity among the three *R. leguminosarum* isolates was evaluated, using six random primers (OPB-05, OPB-07, OPB-10, OPB-12, OPB-18 and OPT-20). Since, three primers (OPB-07, OPB-12 and OPB-18) gave genetic diversity among isolates (Tables 4 and 5). In total, 25 bands were produced, 5 of which were polymorphic. Primers OPB-12 and OPB-18 produced the highest polymorphic bands. The percentage of polymorphic bands ranged from 8.3% to 33.3% with an average of 20%. Number of monomorphic bands range from 1 to 8 with average 4 per primer. Number of unique bands ranged from 1 to 4 with an average of 2.7 per primer, it was observed that most of unique bands scored at F isolate.

Table 2. Protein content and fractions of *R. leguminosarum* isolates.

***R. leguminosarum* isolates**				**Polymorphism**
MW	**M**	**IS**	**F**	
KDa	**Density**	**Density**	**Density**	
200	++	++	++	Monomorphic
150	+	-	+	Polymorphic
120	++	+++	++	Monomorphic
100	-	++	+	Polymorphic
89	+	+	-	Polymorphic
70	+	+	-	Polymorphic
60	-	-	+	Unique
50	++++	++++	++++	Monomorphic
40	-	+	+	Polymorphic
30	-	+	-	Unique
25	++	++	++	Monomorphic
20	++++	++++	++++	Monomorphic
15	+++	+++	+++	Monomorphic
12	+	-	-	Unique
8	-	++	++	Polymorphic
10		12	11	No of polypeptides
89.25		92.27	90.75	Protein content (mg/g)

MW= Molecular weight markers (KDa).
Density band (–) = absent band, (+) = weak, (++) = moderate, (+++) = strong, (++++) = very strong.

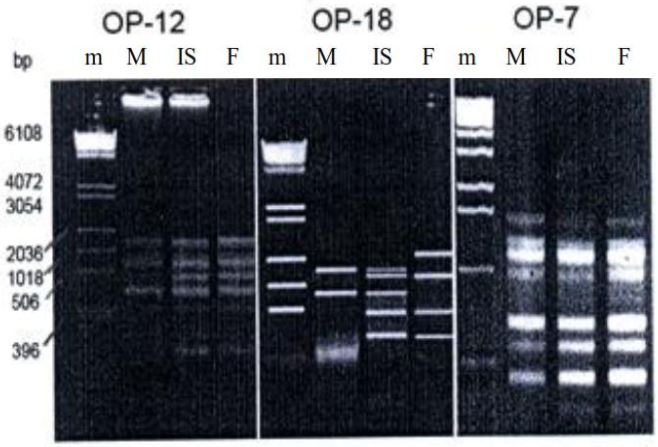

Figure 2. 1.5% agarose gel electrophoresis showing the DNA amplified PCR products of 3 *R. leguminosarum* isolates using 3 random primers.
m = Molecular weight DNA marker.
M, IS, F = *R. leguminosarum* isolates.

Table 3. Peroxidase (PRX) isozyme analyses of variable bands of three *R. leguminosarum* isolates.

No. of band	*R. leguminosarum* isolates			Polymorphism
	M	IS	F	
1	++	+++	+++	Monomorphic
2	-	+	-	Unique
3	++	+++	+	Monomorphic
4	+	+	-	Polymorphic
5	-	++	++	Polymorphic
6	-	++	++	Polymorphic
7	++	-	-	Unique
8	-	-	++	Unique
No of Polypeptides	4	6	5	

Table 4. RAPD amplified bands, polymorphic and genetic markers for *R. leguminosarum* isolates using random primers.

Random primers												
	OPB-07				OPB-12				OPB-18			
R. leguminosarum isolates												
	F	IS	M	Polymorphism	F	IS	M	Polymorphism	F	IS	M	Polymorphism
3000	++	-	++	Polymorphic	-	-	-	-	-	-	-	-
2960	++	++	++	Monomorphic	-	-	-	-	-	-	-	-
2504	++	++	++	Monomorphic	-	-	-	-	-	-	-	-
2296	+++	+++	+++	Monomorphic	-	-	-	-	-	-	-	-
2125	+++	++	++	Monomorphic	-	-	-	-	-	-	-	-
1625	+	+	+	Monomorphic	-	++	+	Polymorphic	++	+	++	Monomorphic
1560	-	-	+	Unique	-	-	-	-	+	+	-	Polymorphic
905	+++	+++	+++	Monomorphic	+++	-	-	Unique	++	+	++	Monomorphic
712	+++	+++	++	Monomorphic	-	++	+	Polymorphic	-	++	+	Polymorphic
510	-	-	-	-	-	+	-	Unique	-	-	-	-
413	+++	+++	++	Monomorphic	++	++	+	Monomorphic	+	++	+	Monomorphic
367	-	-	+	Unique	+	-	-	Unique	-	-	-	-
250	+	-	-	Unique	++	-	-	Unique	+	-	-	Unique
Total No. of fragments	10	8	11		4	4	3		5	5	4	

Table 5. List of primers, No. of bands and polymorphism in each primer.

Primer	No. of Polymorphic bands	No. of Monomorphic bands	No. of Unique bands	Total No. of bands	Polymorphism (%)	Monomorphism (%)	Uniqueness (%)
OPB-07	1	8	3	12	8.3	66.7	25
OPB-12	2	1	4	7	28.6	14.3	57.1
OPB-18	2	3	1	6	33.3	50	16.7
Total bands scored	5	12	8	25	20	48	32

4. Discussion

Protein patterns, peroxides isozymes and DNA fingerprints were used for analysis of somaclonal variations among three *R. leguminosarum* isolates namely M, IS and F (Amer, 2008a; b). They should be independent of *Rhizobium* genetics under environment stress. Whereas, amino acid sequence of polypeptides are depend on nucleotide sequence of their coding genes. Therefore, on electrophoretic analysis of protein and isozymes of three isolates of *R. leguminosarum* approximates the analysis of their genetic variation. The variation of three isolates was

detected via determination of polypeptides by using SDS-PAGE and peroxidase isozymes by using DISC-PAGE. Data presented in table (2) indicated the variation in both the number and density of protein fractions among the three isolates. Also, these results indicated the variation of protein content among the three isolates. This suggests that despite increasing the biodegradation of protein content, new protein types were detected due to the differential response of the three isolates to the effects of the environment stress. The same observation was reported by Swelim, 2005; Aiat (2006); Girgis *et al*. (2008) and Shash (2008). The syntheses of new proteins are due to the interaction between bacteria and environment conditions.

The enzymatic plots and their metabolic pathway are the most important factors affecting ecology conditions. The results showed that the levels of peroxidase among the three isolates were different. The increase in isozymes number plays an important role in the defense mechanism. Also, the increase in the enzyme activity has been detected by conditions in different microorganisms and ecology (Hammerschmidt *et al*., 1982).

DNA prepared from three isolates was found crucial for RAPD-PCR. The yields of DNA were determined by spectrophotometrically as 1.8, 1.9 and 1.75 µg/0.5 g cells of the three isolates M, IS and F, respectively. The PCR conditions for DNA analysis were optimized by investigating each factor individually. The optimized conditions were detailed in materials and methods section. A total of amplified DNA fragments raining in size of 3000 to 250 bp were observed using the three primers and were expressed as polymorphic with 5 bands (20%) monomorphic with twelve bands (48%) and 8 genetic markers (unique) with 32% which were detected among the three isolates. Interesting to note that, the three isolates were significantly varied.

The results of the present study gave preliminary informative DNA-based makers for 3 *Rhizobium* isolates. Also, optimizations of experimental conditions of PCR amplification are a prerequisite for the performance of RAPD analysis. This increased the reproducibility and efficiency of RAPD as a molecular marker technique. The three *Rhizobium* isolates were isolated from faba bean nodules (Amer 2008a; b). However, three random primers gave producible and very stable results peculiar to the same specific from different accessions. The other primers sometimes did not give the exact fingerprints for the 3 *Rhizobium* isolates. During the past years, numerous publications demonstrated the utility of RAPD markers for the analysis of the genetic variability among isolates of bacteria, fungi, actinomycetes and plant populations (perret and Broughton, 1998; El-Dougdoug *et al*., 2007; Swelim, 2005; Girgis *et al*. (2008) and Shash (2008).

The molecular mechanism underlying somaclonal variations have been attributed to chromosome breakage, single base changes in copy number of repeated sequences and alteration in DNA methylation patterns (Kaeppler and Philips, 1993 and Munthali *et al.,* 1996). The polymorphism in amplified products may be either a result from changes in the sequence of the primer binding site (e.g. point mutations) or changes which alter the size or prevent the successful amplification of the target DNA (e.g. insertions, deletions, inversions) as suggested by Rani *et al*. (1995).

It could be concluded that protein, isozymes and DNA fingerprint can be successfully used to detect somaclonal variations among the three *R. leguminosarum* isolates. Numerous researches proved that the sensitivity at protein, isozymes, and DNA analysis were sufficient enough to detect genetic variability in many of bacteria, fungi and actinomycetes (perret and Broughton, 1998; Sharma, 2003 Swelim, 2005; Girgis *et al*. (2008) and Shash (2008).

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