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SEROTYPING OF *Rhizobium leguminosarum* BIOVAR *phaseoli* AND THEIR SYMBIOTIC PERFORMANCE

Rizk, R. Y.

Soil Microbiol. Lab., Sakha Agric Res. Station, Soil Water and Environ. Res. Inst., A.R.C., Giza, Egypt .

ABSTRACT

Ten effective isolates of *R. legumisarum* biovar *phaseoli* (*phaseolus vulgaris L.*) were isolated from 3 governorates of lower Egypt (Kafr El-Sheikh, El Behera and El Gharbia) at different locations during season (2002). All the tested isolates gave positive results when subjected to purify tests.

Screening of the isolates, using intrinsic antibiotic resistance (IAR) method against ampicillin, kanamycin sulphate, rifampicin, streptomycin sulphate, neomycin, dexacillin, erythromycin, terramycin, topramycin and colistin sulphate at low and high concentration and showed variability to other antibiotics tested.

On the basis of serological analysis, the representative isolates of IAR groups are classified into four groups. Comparing IAR identification patterns of the isolates, with serotyping data. It was found that many isolates which showed similar pattern to antibiotic resistance could be considered as one serotype.

The representative strains were investigated for their symbiotic performance under controlled conditions. Results showed that the obtained strains exerted different responses in association with its specific host of *phaseolus vilgaris* var. Giza.

INTRODUCTION

Bean crop (*phaseolus vilgaris* L.) is an important component of the Egyptian diet, the increase in the quality and quantity of its yield is an important national goal .

Many varieties of *phaseolus vulgaris* can obtain much of their N requirement through symbiotic N_2-fixation if the root nodules are formed by effective strains. In these case the host plant provides a home for the bacteria and energy to fix or gather air nitrogen. In return the plant receives fixed nitrogen from the nodules. The fertilizers which supply nitrogen are very expensive especially, in the developing countries, where fertilizers are imported. Thus, alternate sources of N–fertilizer need to be studied. One such alternative is biological nitrogen fixation. Strains of *R. Leguminosarum* biovar *phaseoli* that have dramatic differences in such important traits as host specificity, ineffectiveness and effectiveness are indistinguishable from each other under microscopic observation or cultural features and biochemical tests. However, *Rhizobium* serology has been useful in the evaluation of the taxonomic relatedness among *Rhizobium sp.* (Vincent and Humphrey, 1970; Abdel-Rhim *et.al*, 1978) and their identification when isolated from nodules (Ghobrial *et. al* 1991; 1992). The parallel use of antibiotic marker and serodignosis both relatively stable in themselves, provide a means of confirming the stability of each marker independently in ecological research. Little information is available on the population diversity of symbiotic N-fixing rhizobia specific to this cultivars. In this study, we

assessed the diversity within indigenous rhizobial isolates from different locations in Egypt , using IAR marker and serological diagnosis to follow up their persistence and behavior when introduced into the soil. The study also includes the potential of effective selected rhizobial strains under controlled conditions.

MATERIALS AND METHODS

1- Isolation of the *Rhizobium* strains

Ten rhizobial isolates obtained from root nodules of bean grown in 3 governortes in lower Egypt(Kafr El-Shekh, El-Behera and El Gharbia). Healthy nodules were separated from the bean root, immersed for 10 seconds in 95% ethanol, then soaked in 93% sodium hypochlorite solution for 3 minutes and rinsed several times with sterilized distilled water. Individual nodules were crushed in sterile distilled water/ml. One loopful of each nodule suspension was streaked into plates of yeast extract mannitol agar (YEMA) medium (Vincent,1970) containing congo red at a final concentration of 25 $mg/1^{-1}$. Subcultring occurred only once during the experiment and single colonies were selected and each isolate was restreaked to purify(Somasegaran and Hoben, 1994). The bacterial isolates were stored on YEMA slant tubes for further investigation. The screening procedure, based on the assessment of the plate count of the surviving
cells (colony forming units, CFU) on (YEMB).

2- Intrinsic Antibiotic Resistance (IAR) characteristics of *R. leguminosarum* bivar *phaseoli* isolates

Ten rhizobial isolates, which formed nodules on bean plants were characterized using intrinsic antibiotic resistance (IAR) patterns. The antibiotics used and their concentrations, $\mu,g\ ml^{-1}$ were Ampicillin (AM,30) Kanamycin sulphate (KN,10), rifampicin (RE,5), streptmycin sulphate (ST,20), neomycin (NO 20) amoxycillin (AX, 25), Erythromycin (ER,10), Terramycin (TR,5), topramycin (TP, 10) and colistin sulphate (CT, 50). The antibiotic solutions were filter sterilized (0.20 mm). YEAM (Vincent, 1970) plates separately containing the antibiotics tested were used to determine the resistance level of the tested isolates to each of the antibiotics under investigation. Each rhizobial isolate was streaked on YEMA plates supplemented with each antibiotic tested. Three plates of each antibiotic were used for each rhizobial isolate and incubated for 7 days at 28°C and the growth was scored by visual inspection as (+) for growth and (-) no growth.

3- Antigen preparation, immunization and serotyping of the selected isolates.

Four isolates (R.P.V.1, R.P.V.4 ,R.P.V.6, and R.P.V.5) representing the IAR group of a *Rhizobial* culture was maintained on 7 liters of Phaseolus medium in 10 liters flasks and aerated by bubbling sterilized air, or in 500 ml conical flasks placed on rotary shaker. Cultures were harvested after 6 days by centrifugation at 8.000 r.p.m. the precipitated cells were washed several times in a sterilized physiological solution (0.85% NaCl), then stored at

freezing temperature until usage. Antigens applied-for *in vivo* immunization were prepared by carefully adding 10 ml of Freund's complete adjuvant drop to heavy cell suspension (Kabat and Mayer, 1971). The mixture was continuously stirred in one direction until a white colloidal paste was obtained. Antigens for in vitro serological reaction were obtained by adding 16 grams of fine washed sand to 8 grams of washed cells. The mixture was then crushed thoroughly in a mortar submerged in an ice box, then centrifuged at 4000 r.p.m., and subsequently the precipitates were discarded. The antigens were kept in tubes at 0C°. Before use the antigens protein content was determined calorimetrically by a Biuret reagent (Kabat and Mayer, 1971).

Rabbits 2-3 kg. in weight were immunized by a weekly injection with 1 ml. of the antigens. A blood sample was taken from the lateral ear vein, 7 days after each injection, and the antiserum was tested against the homologous antigens by the matrix technique (Jokay and Karczage, 1968). When the maximal precipitin bands were obtained, a cell suspension in a physiological solution without adjuvant was injected subcutaneously. Bleeding was carried out 8 days after the last injection, and the blood was incubated for 3 hours, then stored overnight at 4C° and the separated antiserum was kept in vials at 0C°. Rabbits immunized by the tested , Rhizobia received 12 to 14 injections. The tested isolates that represent IAR groups designated A, B, C, and D were allowed to react with their homologous as well as their heterologous antigens through agglutination and a double diffusion test via matrix technique (Jokay and Karczage, 1968).

Evaluation of symbiotic performance of *R. leguminosarum* biovar *phaseali*

Infectivity and efficiency of the isolates represent the four IAR groups were evaluated in association with bean (*phaseolus vulgaris* L.) C.V. Giza using leonard-jar system (Vincent,1970). Number and dry weight of nodules, shoot dry weight and plant nitrogen content were determined after 45 days of sowing (Ward and Johnston, 1962).

RESULTS AND DISCUSSION

Antibiotic marker

All of the tested isolates were intrinsically sensitive to (rifampicin 5 μ gml^{-1} and terramycin 5 $\mu g\ ml^{-1}$). On the other hand, the four isolates (R.P.V.5, R.P.V.7, R.P.V.8 and R.P.V.10) were intrinsically sensitive to seven antibiotics tested. However, the pattern of the intrinsic antibiotic resistance showed that the isolates were divided into four groups (Table 1). The isolates of each group showed the same pattern as IAR. However, group A includes (R.P.V.1 and R.P.V.3) showed resistance to seven antibiotics out of ten antibiotics tested. These results may indicate the similar genetic background of the isolates of each group irrespective of their difference in site of isolation and climatic region. Intrinsic antibiotic resistance profile was used to identify strains of *R. leguminosarum* bv. *Viceae* (Josey *et al.*, 1979;Brockaman *et al.*, 1989).

Table (1) : Variation in intrinsic antibiotic resistance (IAR) among isolates of rhizobia nodulating bean

Groups	Rhizobial isolates	AM 30 $\mu m1^{-1}$	KN 10 $\mu m1^{-1}$	RF 5 $\mu m1^{-1}$	ST 20 $\mu m1^{-1}$	NO20μ $m1^{-1}$	AX 25 $\mu m1^{-1}$	ER 10 $\mu m1^{-1}$	TR 5 $\mu m1^{-1}$	TP 10 $\mu m1^{-1}$	CT 5 $\mu m1^{-1}$
A	R.P.V. 1	+	+	-	+	+	+	+	-	+	-
A	R.P.V. 3	+	+	-	+	+	+	+	-	+	-
B	R.P.V. 4	-	+	-	+	+	+	-	-	+	+
B	R.P.V. 9	-	+	-	+	+	+	-	-	+	+
C	R.P.V. 2	+	-	-	+	+	-	+	-	-	+
C	R.P.V. 6	+	-	-	+	+	-	+	-	-	+
D	R.P.V. 5	+	+	-	-	-	-	-	-	+	-
D	R.P.V. 7	+	+	-	-	-	-	-	-	+	-
D	R.P.V. 8	+	+	-	-	-	-	-	-	+	-
D	R.P.V. 10	+	+	-	-	-	-	-	-	+	-

AMPICILLIN (AM), Kanamycin (KN), rifampicin (RF), streptomycin (ST), neomycin (NO), amoxycillin (AX), erthromycin (ER), terramycin (TR), topracin (TP) and colistin sulphate (CT).

On the other hand, (Young and Chao, 1989) reported that both the fast and slow growing strains of rhizobia showed wide variability in resistance to antibiotics. Our results suggests that IAR characteristics can be used as a complementary tools in conjunction with other serological methods to identify *Rhizobium* strains.

Agglutination and number of precipitin lines formed in double diffusion tests between cross-reactive antigens of the tested isolates.

This experiment was carried out to investigate the efficiency of serological methods in differentiation between different isolates of *R.leguminosarum* biovar *phaseoli* behaved differently in their response to antibiotics resistance. Therefore, immunization of animals with antigens extracted from four isolates (represent 4 IAR group) to raise antibodies, and the qualitative analysis of the cross reactive antigens of the antibodies, so raised, with antigenic materials of the whole cell as well as their extractions. This was accomplished by agglutination and double diffusion test. Data presented in (Table 2) indicate that the antigens tested gave positive reaction with homologous antigens, while with hetrologous antigens, gave negative results.

Table (2): Cross agglutination tested between the isolates of *R. leguminosarum* biovar *phaseali*

Isolates	R.P.V. 1 (A)	R.P.V. 4 (B)	R.P.V. 6 (C)	R.P.V. 5 (D)
R.P.V. 1	+	-	-	-
R.P.V. 4	-	+	-	-
R.P.V. 6	-	-	+	-
R.P.V. 5	-	-	-	+

The precipitin patterns obtained from double diffusion testes conducted between the antisera of the tested isolates and their respective and irrespective antigens are presented in (Table 3). When the precipitin bands are matched, the following could be concluded: (a) enumeration of the precipitin band formed, revealed that the highest number of precipitin lines were developed when every antisera was allowed to react with its homologouse antigens as they gave 9, 11, 12 and 14 bands for isolates R.P.V. 1, R.P.V.4, R.P.V.6 and R.P.V.5 respectively, (b) occurrence of certain common antigens between all the tested isolates, as they shared common precipitin lines, when every antisera was subjected to react with the heterologous antigens of other tested isolates and (c) the common precipitin band developed in the homologous reactions ranged from 4-8 lines as shown in (Table 3). Results obtained from agglutination and double diffusion tests indicated that the tested isolates could be related to four different serogroup, according to their antigenic structure, which are compatible with their IAR pattern.

Therefore, the results of serological tests could be considered, as additive criterion strengthen the aforementioned results obtained from the IAR pattern. The results also proved that certain common antigens existed in all the tested isolates as they belong to one spices of *R. leguminosarum*

biovar *phaseoli*. Such findings also correlated with that found by (Chanway and Holl, 1986) who showed that serology is less variable than IAR when strains of *R. trifolii* are identified.

Table (3): The number of preciptitin band formed in cross reactions between the isolates of *R. leguminosarum* biovar *phaseoli*

Isolates	R.P.V. 1 (A)	R.P.V. 4 (B)	R.P.V. 6 (C)	R.P.V. 5 (D)
R.P.V. 1	9	6	5	5
R.P.V. 4	6	11	7	5
R.P.V. 6	8	7	12	6
R.P.V. 5	5	4	4	14

Symbiotic performance of selected rhizobial strains

1- Number and dry weight of nodules :

Number and dry mass of nodules have often bean employed for indirect measurement of N_2-fixation (Weber, 1966 and Westermann and Kolar, 1978). The effect of inoculation on nodule number as observed after 40 days from planting time (Table 4) was very clear by bean plats variety Giza when inoculated with any of the four rhizobia strain applied, strain R.P.V.5 produced the highest mean number of nodules lowest with (33.7/plant), strain R.P.V.6 was the lowest (24.6/plant) with regard to dry weight of nodule, data in (Table 4) clarified that the total mass of nodules followed a pattern similar to that of nodule number.

Table (4): Effect of inoculation with *R. leguminosarum* biovar *phaseoli* on number (nod / plant), dry weight of nod. (g / plant), dry weight of shoots (g / p) and N. content of shoots (mg / p) of bean plants

Treatments	No. of nod. / p	D.W. of nod. (g / p)	D.W. of shoots (g / p)	N. content (mg / p)
Untreated	0	0	3.26	24.45
R.P.V. 1	30.5	0.172	4.87	62.33
R.P.V. 4	26.9	0.155	4.56	58.31
R.P.V. 6	24.6	0.148	4.25	54.40
R.P.V. 5	33.7	0.182	5.11	66.43

2- Dry matter of shoots and their total nitrogen content:

Dry matter production of bean cultivar Giza was strongly influenced by nodulating strains as shown by striking differences between inoculated and control treatment (Table 4). The effect of inoculation was clearly reflected on the accumulation of dry weight of plant material and N-content strain R.P.V.5 gave high dry weight and N-content of shoots being 5.11 g/plant and 66.43 mg/plant and 66.43 mg/plant. The other inoculated treatments with strain R.P.V.1 , R.P.V.4 and R.P.V.6 showed variable amount of dry matter and N-content of shoots ranged between 4.25 and 4.4.87 and content of shoots between 54.4 and 62.33 mg/plant.

In general, results obtained from – bean - *Rhizobium leguminosarum* bivar *phaseoli* associations indicate that improvement of nitrogen fixation could be achieved by selection of highly efficient strains isolation from natural environment.

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تصنيف ريزوبيا الفاصوليا سيرولوجيا وتقييم كفاءتها التكافلية

رزق يوسف رزق

المعمل البكتيرى بمحطة البحوث الزراعية بسخا – معهد بحوث الأراضى والمياه والبيئة – الجيزة – مصر

تم الحصول على (١٠) عزلات من ريزوبيا الفاصوليا موسم ٢٠٠٢ من عدة مواقع مختلفة بمحافظات الوجه البحرى / ولقد ثبت نقاوة تلك العزلات المتحصل عليها باستخدام اختبارات النقاوة المختلفة.

وبدراسة المقاومة الذاتية للعزلات المنتقاه (IAR) لعشر مضادات حيوية هى الأمبسلين ، الكانامبسين ، ريفامبسين ، الاستربتوميسين ، نيوميسين ، الاموكسيسللين ، ارثيروميسين ، الترامبسبن ، التوبرامبسين والكوستين وجد أنها تقع فى أربعة مجموعات مختلفة.

وقد أوضح التصنيف السيرولوجى لتلك المجموعات الأربعة المتحصل عليها توافقا مع نتيجة المقاومة للمضادات الحيوية (IAR). وبناء على ذلك أمكن تقسيم العزلات العشرة للفاصوليا إلى أربعة مجموعات (سلالات) مختلفة سيرولوجيا.

وتم أخذ سلالة ممثلة لكل مجموعة وتقييم كفاءتها التكافلية تحت ظروف معقمة باستخدام ليونارد جار وتقدير وزن وعدد العقد الجذرية الجافة والوزن الجاف للسيقان وتقدير المحتوى الكلي للنيتروجين في السيقان، وأظهرت النتائج وجود فروقات بين السلالات الأربعة.