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Ecological Studies on Soil Indigenous Rhizobia in Egypt. 3-Intrinsic Antibiotic Resistance (IAR) for Differentiation of Rhizobia

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ANTIBIOTIC marking is generally used in following up the persistence of induced rhizobial strains to soils. As well known maximizing symbiotic N_2 fixation requires a great understanding of the ecology of rhizobia. One of the major limitations in study of this symbiosis is the difficulty in recognizing strains of rhizobia in their natural habitats. Intrinsic antibiotic resistance (IAR) of 131 *Rhizobium* isolates 381 belonging to clover, 21 to chickpea and 29 to pea towards low, moderate and high concentrations of 13 antibiotics was investigated. All isolates possessed IAR character but the number of antibiotics resisted varied and the resistance ability of most isolates decreased with the increase of antibiotic concentration in the culture medium (YMA). Use of low or moderate concentrations gave more reliable information about the IAR character. Gentamycin and Kanamycin have the most suppressive effect on the three species of tested rhizobia, *R. leguminosarum* bv. *trifolii*, *R.* spp. and *R. leguminosarum* bv. *viceae*. Chickpea *Rhizobium* was also affected by streptomycin at the three levels of concentration, while pea isolates severely affected also by polymyxin especially at moderate and high levels of antibiotic since none isolates could grew on the medium. Spectinomycin was of moderate effect on growing of pea *Rhizobium*.

Keywords: Resident rhizobia, Intrinsic antibiotic resistance (IAR), Clover, Chickpea and Pea rhizobia.

Intrinsic antibiotic resistance (IAR) is an important character found in many groups of microorganisms which is known to play a role in different kinds of environmental activities. Many of microflora posses the ability of withstand the lethal or suppressive effect of various antimicrobial substances, including antibiotics, which prevail in microsites of the soil. Therefore, antibiotic marking is generally used in following up the persistence of induced rhizobial strains to soils. As well known, maximizing symbiotic N_2 fixation requires a great understanding of the ecology of rhizobia systems (Kremer & Peterson, 1982). Several techniques have been suggested for rhizobial strain identifications such as biochemical and metabolic tests (Graham & Parker, 1969), genetic markers (Schwinghamer & Dudman, 1973), and using of strain specific antisira (Abd El-Maksoud & Somasegaran, 1988). Antibiotics have also been used in selective

media for rhizobia recognition (Knigsley & Bohlool,1983; Muller *et al.*,1988 and Josic *et al.*, 2002). Shekhar (1997) suggested that antibiotic resistance–marked strains should be compared with wild-type parents before being used as monitors of parental strain survival. Schwinghamer & Dudman (1973) studied the resistance to the antibiotic spectinomycin as possible marker to supplement streptomycin resistance in ecological study with rhizobia. Single step spontaneous mutants resistant to high levels of spectinomycin were isolated from 8 effective strains representing 4 species of *Rhizobium* , they concluded that there was no evidence of cross resistance to streptomycin, and streptomycin resistant mutants were not cross resistant to streptomycin. Josey *et al.* (1979) studied the variation of IAR of eight antibiotics for identifying characteristics of 26 *R. leguminosarum* strains. They mentioned that the IAR of each strain was a stable property by which rhizobia isolated from root nodules of inoculated *Pisum sativum* could be recognized. Gupta & Kalra (1987) studied a number of UV induced antibiotic resistant mutants of *R. leguminosarum* bv. *trifolii* IIB. They found that all mutants were resistant to ampicillin, erthromycin and nalidixic acid except one mutant, which was sensitive to erythromycin. The resistance to chloramphenicol and kanamycin was found only in one mutant, whereas none of mutants were found to be resistant to gentamycin. Jarek (1989) reported that the most inhibiting effect on the *R. leguminosarum* strains was observed with gentamycin and they were most resistant to penicillin. In this respect, Moawad & Bohlool (1992) used seventy six isolates of *Leucaena* plants of various genotypes growing in a wide range of soil types and climatic regions, they found that IAR patterns for eight antibiotics were helpful for selecting the strains for immunization research. Therefore, it was suggested to study the IAR property to be included together with the other biochemical characteristics carried out for the collection of *Rhizobium* cultures isolated from clover, chickpea and peas obtained from different localities of Egypt.

Material and Methods

Resident rhizobia capable of nodule formation on root of clover (*Trifolium alexandrinum*), chickpea (*Cicer arietinum*) and pea (*Pisum sativum*) were isolated from different locations included 10 governorates in the Nile Delta and Upper Egypt. A survey of 31 sites was carried out during the winter season at plant age of 2-3 months. A total of 131 native rhizobial isolates (81 from clover, 21 from chickpea and 29 from pea plants) was tested. Rhizobial isolates were investigated for their IAR patterns towards 13 antibiotics using the method given by Moawad & Bohlool (1992).

The antibiotics used and their tested concentrations at 3 levels, low, medium and high are shown in Table 1. Stock solutions were prepared by dissolving 50 mg active ingredient (*a.i.*) of each of the antibiotics in 5 ml sterile distilled water. Naldixic acid was dissolved in (1M) NaOH sterile solution, while erythromycin was dissolved in 95% ethanol. Appropriate volumes of each of the stock solutions were separately added to yeast extract mannitol agar (YMA) medium previously melted and cooled to about 5°C. After mixing, the medium

was poured in Petri dishes and spot inoculation with 48 hr – old of tested rhizobial isolates was carried out using a sterile metal replicator (Somasegaran & Hoben, 1994).The plates were incubated at 28 °C for 48 hr. Visual inspection of bacterial growth was done and compared with the corresponding growth antibiotic-free control plates. The test was carried out in duplicates.

TABLE 1. Antibiotic concentrations.

No.	Antibiotic		Concentration mg.l^{-1}		
			low	medium	high
1	Ampicillin	Amp.	10.00	20.00	40.00
2	Chloramphenicol	Chl.	2.00	10.00	20.00
3	Erthromycin	Ery.	1.25	2.50	10.00
4	Gentamycin	Gen.	5.00	10.00	20.00
5	Kanamycin	Kan.	5.00	10.00	20.00
6	Nalidixic	Nal.	5.00	10.00	20.00
7	Neomycin	Neo.	2.50	5.00	10.00
8	Polymyxin	Pol.	5.00	10.00	20.00
9	Rifampicin	Rif.	1.00	3.00	5.00
10	Spectinomycin	Spe.	5.00	10.00	20.00
11	Streptomycin	Str.	1.00	2.50	10.00
12	Tetracyclin	Tet.	1.00	2.50	5.00
13	Vancomycin	Van.	1.25	2.50	5.00

Results and Discussion

A total number of 131 *Rhizobium* isolates, 81 belonging to clover, 21 of chickpea and 29 of pea was assessed for their IAR patterns against 13 different antibiotics using 3 levels of concentrations low, medium and high as shown in Table 2. Distinct differences in potentiality of resistance among the examined rhizobial isolates towards the number and concentration of antibiotics was recorded. Obviously, all of the 81, 21 and 29 rhizobial cultures isolated from nodules of clover (Fig. 1), chickpea (Fig. 2) and pea (Fig. 3) plant, respectively, showed a remarkable ability to resist the effect of many of the tested antibiotics. As expected, the ability of most isolates to resist the tested antibiotics decreased with the increase of the concentration given in the culture medium since, the high concentrations were more suppressive for bacterial growth. In other words, all bacterial isolates could resist one or more up to the 13 antibiotics when applied in low concentration, but at moderate and high concentrations all isolates were adversely affected and a remarkable drop in the number of resisted cultures was recorded. For example, at low concentration ,out of the 81 clover *Rhizobium*, 42, 23 and 16 isolates (51.9, 28.4 and 19.8 %), respectively tolerated from 9 to 13, from 4 to 8 and from 1 to 13 antibiotics. Whereas, at moderate antibiotic concentration, the corresponding numbers were 22, 38 and 21 (27.2, 46.9 and 25.9%), but at the high concentration the number was 0, 42 and 39 (0.0, 51.9 and 48.1%) , respectively. These data are in accordance with those obtained by Glynn *et al.* (1985). A more or less similar trend of results with some little

variations could be found for chickpea rhizobia. Such result actually supports previous finding of Kingsley & Bohlool (1983). It was also found by Garg *et al.* (1985) that, of 210 wild type isolates of chickpea rhizobia, only 15 were resistant to one or more of seven antibiotics at a concentration of 5 mμ g/ml. There of the antibiotic-resistant strains were as effective in nitrogen fixation as a standard antibiotic-sensitive strain. In this respect, Turco & Bezdicek (1987) obtaind similar results through their study on pea rhizobia. The present result indicates that the use of relatively low or moderate concentrations of the antibiotics give a more reliable information about the IAR and how far is the range of resisted antibiotics a *Rhizobium* strain can tolerate and this could be very helpful in the ecological and diversity studies of rhizobia as mentioned by Issa & Wood (1995). Comparison between the 13 antibiotics in their effect on the total 131 rhizobial isolates revealed that both Kanamycin and Gentamycin have a distinct suppresive effect on the three species of rhizobia. Since only 12, 2 and 6 isolates (14.8, 9.5 and 20.7%) of clover, chickpea and pea rhizobia, respectively, resisted Kanamycin at low concentration. No growth was obtained under the moderate and high concentration for chickpea and pea rhizobia whereas only one isolate of clover could survive at the high concentration of that antibiotic. A number of 12 clover isolates (14.8%) could resisted Gentamycin at low concentration, but all the 3 levels of concentration were acute lethal for the two other species of chickpea and pea. On the other hand, Rif., Amp., Chl., Ery., Tet., and Van., had the least antibacterial effect since 59, 60, 67, 68, 69 and 70 isolates of clover *Rhizobium* (72.8, 74.1, 82.7, 82.7, 84.0 and 86.4 %) gave a positive IAR towardes them. The 13 antibiotics could be arranged in descending order as follows Van.> Tet.> Ery.> Chl.> Amp.> Nal.> Rif.> Pol.> Neo.> Spe.> Str.> Kan.> Gen.

TABLE 2. Intrinsic antibiotic resistance (IAR) pattern for rhizobial isolates.

Antibiotics	Concentrations								
	low	medium	high	low	medium	high	low	medium	high
	R. leguminosarum bv. *trifolii* Clover (81isolates)			*Rhizobium* spp. Chickpea (21 isolates)			*R. leguminosarum* bv. *viceae* Pea (29 solates)		
Ampicillin Amp.	60	51	42	21	19	18	29	28	27
Chloramphenicol Chl.	67	43	12	21	20	8	28	28	27
Erthromycin Ery.	67	66	50	21	21	21	29	29	29
Gentamycin Gen.	12	8	0	0	0	0	0	0	0
Kanamycin Kan.	12	12	1	2	0	0	6	0	0
Nalidixic Nal.	55	49	21	18	16	8	29	29	28
Neomycin Neo.	43	21	6	21	4	0	19	5	4
Polymyxin Pol.	47	37	10	21	13	4	6	0	0
Rifampicin Rif.	59	35	8	21	18	1	28	25	12
Spectinomycin Spe.	42	26	2	21	13	1	14	8	4
Streptomycin Str.	24	20	9	7	3	2	18	16	12
Tetracycline Tet.	68	65	65	21	20	20	23	22	22
Vancomycin Van.	70	68	64	21	21	21	28	28	26

*Number of isolates with IAR against referred concentrations of different antibiotics (see Table 1).

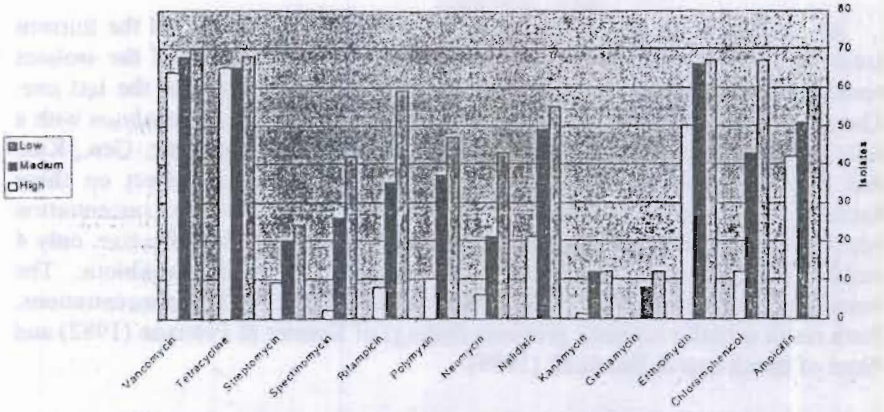

Fig. 1. Clover isolates vs. antibiotic concentrations.

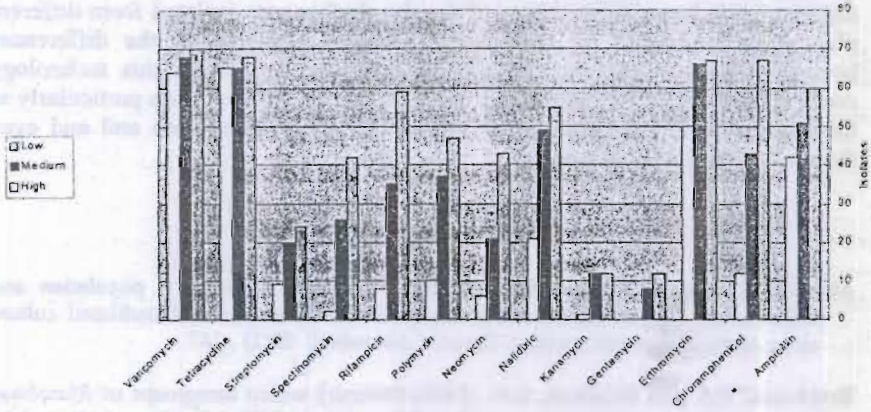

Fig. 2. Chickpea isolates vs. antibiotic concentrations.

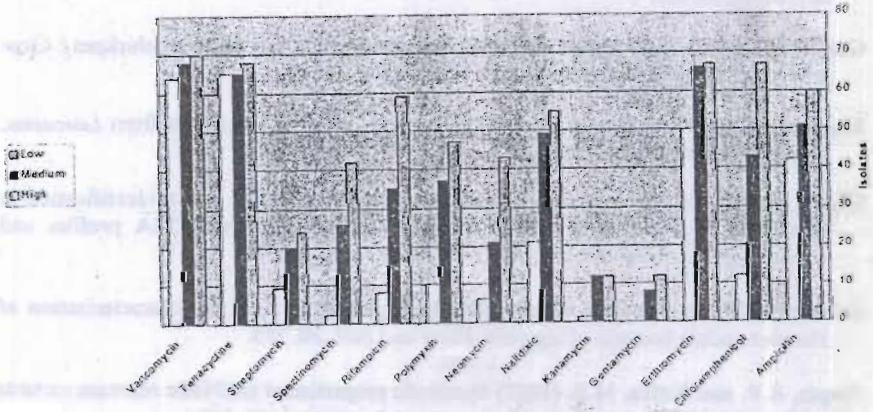

Fig. 3. Pea isolates vs. intibiotin concentrations.

All isolates of chickpea *Rhizobium* could resist successfully, all the thirteen antibiotics, except Gen., Kan. and almost Str. Since one third of the isolates could grew on medium containing 1 $mg.l^{-1}$ (low concentration) of the last one. The same trend was observed in the intrinsic resistance of pea *Rhizobium* with a little variation. Since, in addition to the three-suppressive antibiotic, Gen., Kan. and Pol., there was another one, Spe. which has moderate effect on these bacteria. About half of total isolates (48.3%) could grew at the low concentration whereas the moderate and high concentrations were more lethal effective, only 4 isolates (13.8%) could survive at high concentration of that antibiotic. The remaining antibiotics were of light effect particularly at the low concentrations. Such result actually supports previous findings of Kremer & Peterson (1982) and those of Brockman & Bezdicek (1989).

In general, data obtained from this work clearly show that certain concentration of antibiotics used in the medium (YMA) were suitable for differentiation between members of the rhizobial groups isolated from different plant hosts nodulated by indigenous isolates. Depends on the differences between strains of rhizobia towards the different antibiotics, this technology could be successfully employed for the field of ecological studies particularly in the recovery and enumeration of rhizobia cells introduced into soil and even those of soil indigenous strains.

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دراسات بيئية على الريزوبيا المتوطنة بالأراضي المصرية.
٣- تصنيف الريزوبيا باستخدام القدرة الذاتية (الكامنة) على مقاومة المضادات الحيوية

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كما هو معروف فإن عملية التثبيت البيولوجى للنيتروجين عن طريق المعاشرة بين النبات البقولى وميكروب الريزوبيا يحتاج إلى فهم كامل للعوامل البيئية المحيطة بها. ويعتبر التعرف على السلالات ذات الكفاءة العالية فى تثبيت النيتروجين وعزلها من هذه التربة ومدى تأثرها بالظروف البيئية المحيطة بها من أملاح ومبيدات ومضادات حيوية(تفرزها بعض الميكروبات الأخرى) أحد التحديات التي تواجه مثل هذه الدراسة . وتعتبر القدرة الذاتية (الكامنة) لميكروب الريزوبيا على مقاومة التركيزات المختلفة من المضادات الحيوية إحدى التكنولوجيات التي تساعد علي هذه الدراسة، حيث تمكن من عزل و متابعة الميكروب فى التربة .

أجريت هذه الدراسة على ثلاثة أنواع من الريزوبيا شملت تلك الخاصة بنبات البرسيم ، الحمص و البسلة والمعزولة من على جذور هذه النباتات فى عدد ٣١ موقعا موزعه على ١٠ محافظات على مستوى الجمهورية. وكان عدد العزلات الكلى ١٣١ عزلة منها ٨١ خاصة بالبرسيم ، ٢١ بالحمص و ٢٩ عزلة من البسلة .

استعملت ثلاثة تركيزات (منخفضة ، متوسطة ، عالية) لكل من ثلاثة عشر من المضادات الحيوية وقد اختلفت هذه التركيزات فيما بين المضادات تبعا لنسبه المادة الفعالة وقد وجد أن جميع العزلات قد تميزت بقدرتها على تحمل تركيز أو اكثر من بعض هذه المضادات الحيوية ، وقد اختلفت العزلات للنباتات الثلاث فيما بينها فى عدد المضادات التى أمكن تحملها وكذلك كان الحال بالنسبة للتركيزات المستعملة فيها. كما كانت التركيزات المنخفضة والمتوسطة أقل فى تأثيرها على الميكروب بالطبع عن التركيز العالى والذى أدى إلى عدم نمو أى من العزلات فى بعض المضادات . وقد وجد ان مضاد Kanamycin و Gentamycin هما اشد هذه المضاد الحيوية تحت الدراسة تثبيطا لنمو ميكروب الريزوبيا بأنواعه الثلاث ، فى حين كانت مضادات Vancomycin ,Tetracyclin , Chloramphenicol, Erythromycin Ampicilin أقلها تأثيرا على نمو الريزوبيا عامة، بينما كانت المضادات الأخرى ذات تأثير معتدل تباين باختلاف الميكروب والتركيز المستعمل من المضاد الحيوي .

ويمكن من الناحية التطبيقية استخدام هذه التكنولوجيا بنجاح فى الدراسات البيئية الخاصة بميكروب الريزوبيا تحت ظروف التربة المصرية .