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Ecological Studies on Rhizobia Indigenous to Soils in Egypt 2. Pesticides Tolerance

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THE LEGUME–*Rhizobium* symbiosis is a highly integrated and self-regulating process. The potential influence of hazardous agrochemicals including fungicides and herbicides, on the efficacy of rhizobia was studied for three legume crops, clover, chickpea and peas. Isolates were examined for tolerance towards different concentration of two herbicides (Cotran and Egran) and two fungicides (Dithane and Vitavax). Out of the 81 clover *Rhizobium* isolates, 14 (17.3%) were very sensitive to Cotran while 61 isolates (75.3%) tolerated up to 1000 mg a.i.L^{-1}.The remaining 6 isolates showed variable responses. Almost similar results were recorded for Egran. Clover *Rhizobium* grew in presence of high concentration of Dithane, where 71 isolates (87.7%) developed on YMA medium with 1000 mg a.i.L^{-1}. Only 8 isolates were very sensitive. Vitavax was more suppressive to clover *Rhizobium*. Few isolates tolerated 1000 and 500 mg.L^{-1} (5 and 2, respectively) , while 17 isolates failed to grow in the presence of any tested concentration, remaining isolates showed variable resistance towards, moderate concentrations of the fungicides. All chickpea and pea rhizobial isolates tolerated the maximum concentrations of Cotran and Dithane (1000 mg.a.i.L^{-1}), while Vitavax proved also to be rather suppressive for these bacteria.

Keywords: *Rhizobium* spp., Legume plants, Herbicides (Cotran, Egran), Fungicides (Dithane, Vitavax).

At the beginning of the last century, the vast development of industrial chemistry created different agrochemical compounds for use as fertilizers, pesticides, soil conditioners and phytohormones. The extensive use of such chemicals, for increasing food production for sustainable development, was the main reason for studying these important components. Knowledge of the effects of pesticides on rhizobia and symbiotic nitrogen fixation is important to avoid damage and so that less harmful pesticides could be selected. According to Brooks (1972) and

Dunfield *et al.*, (2000), the concentrations of systemic fungicides on seeds and young plants are applied at rates of a few hundred mg. to 2.0 g kg^{-1}. In contrast, herbicides are used in a dosage of 1.0 kg ha^{-1} and moving in the soil at a depth of 5cm may reach a concentration of only 2.0 mg kg^{-1} (Rensburg *et al.* 1984). In this respect, Martensson (1992) mentioned that herbicides were most harmful to symbiotic N_2 fixation process, with injuries occurring at levels that are 1/10-1/10000 of recommended application levels. It was also found that, the higher herbicide concentrations generally reduced shoot dry weight of legume plants, the number of soil bacteria and fungi, as well as nodule numbers (Gurcharan *et al.*, 1994). Heinonen-Tanski *et al.* (1982) reported that insecticides commonly used are usually applied at small amounts. They also added that, the herbicides and insecticides inhibited the growth of rhizobia to a lesser extent than fungicides. On the other hand, in a survey study on the effects of pesticides on the growth and symbiotic properties of *Rhizobium* spp. Madhavi *et al.* (1993) demonstrated that pesticides generally affected the dry weight and total N content of inoculated plants. The reduction that occurred was in the following order: herbicides > fungicides > insecticides. About the time of application, Strzelec and Martyniuk (1994) stated that the highest numbers of nodules $plant^{-1}$ and maximum seed yield were obtained when fungicides were applied immediately before sowing seeds. Pre-treatment with *Rhizobium*, reduced the number of nodules on roots when fungicides were applied before *Rhizobium* inoculation, and seed dressing with fungicides reduced the growth rate of three strains of *R. leguminosarum* bv. *trifolii* and four strains of *R. meliloti* on YEMA medium and decreased the size and number of colonies. Recently, Feng *et al.*, (1998) found that Captan and Thiram (fungicides) significantly increased chickpea seedlings emergence compared to controls.

In this study we examined the influence of potentially hazardous agrochemicals including fungicides and herbicides on growth and survival of rhizobia on solid growth medium.

Material and Methods

Rhizobia is able to form nodules on the root of clover (*Trifolium alexandrinum*), chickpea (*Cicer arietinum*), and pea (*Pisum sativum*) were isolated from Egyptian soils from 10 governorates (Table 1). During the winter season, plant samples were taken for characterization of root nodules and isolation of harbored rhizobia. In the laboratory, soil samples were thoroughly mixed, used for estimating soil chemical properties (Black, 1982), and the most probable number (MPN) of the selective *Rhizobium* using technique of plant infection test (Somasegaran and Hoben, 1994). Thirty-one soil samples were investigated, representing 26, 2 and 3 of fields cultivated with clover, chickpea and pea, respectively.

Isolation and identification of representative indigenous rhizobia

Within 24 hr from plant sampling, rhizobia were isolated from nodules of the 3 legume plants used yeast mannitol agar (YMA) plates containing congo red. Bacterial cultures were purified by streaking and maintenance of pure culture was achieved through subsequent culturing (Somasegaran & Hoben, 1984).

TABLE 1. Soil analysis, nodule properties on standing legumes and *Rhizobium* MPN

Governorates and Location	Soil analysis						Nodule characteristics		MPN $\times 10^3$ g^{-1} soil
	PH [1]	EC 2 $dS.m^{-1}$	TSS [3] %	Organic C%	Total N%	C/N ratio	Nodule [4] plant^{-1}	Size & colour [5]	
Clover									
1. El-Behira									
Abu-Hummous	7.87	0.45	0.012	1.15	0.21	5.47	28	S,P	1.7
Damanhur	7.91	0.27	0.007	1.02	0.14	7.28	13	S,P	2.1
Itayai el-Barud	7.19	0.25	0.007	1.12	0.10	11.2	75	S,P	17.0
2. El-Gharbia									
Kafr El-Zayat	8.05	0.45	0.012	1.35	0.21	6.42	48	S,W	17.0
Tanta	7.82	0.35	0.009	1.02	0.17	6.00	91	S,P	24.0
3. El-Menofia									
Berkt-el-Sab	7.82	0.42	0.011	1.26	0.26	4.85	102	L,P	21.0
Quweisna	7.65	0.25	0.007	1.11	0.13	8.54	85	S,P	17.0
4. El-Kalioubia									
Benha	7.72	0.35	0.009	1.02	0.10	10.20	10	S,P	0.8
5. Giza									
Tanash	8.23	0.36	0.010	1.14	0.23	5.00	41	S,P	0.8
El-Aiyat	7.94	0.33	0.009	1.41	0.18	7.83	21	S,W	17.0
El-Saff	7.97	0.36	0.010	1.17	0.16	7.31	15	S,W	0.10
6. El-Minia									
Deir-Mowas	7.83	0.42	0.011	1.08	0.20	5.4	45	S,P	12.0
7. Assiut.									
Sedfa	7.90	0.33	0.009	1.13	0.15	7.53	87	S,P	140.0
8. Sohag									
Tahta	8.02	0.40	0.011	1.13	0.29	3.89	20	S,P	2.0
Sohag	7.33	0.26	0.007	0.88	0.19	4.63	26	L,P	1.4
Girga	7.76	0.23	0.006	1.24	0.15	8.26	18	L,P	1.2
El-Balyana	8.01	0.17	0.005	1.1	0.21	5.24	45	L,P	17.0
9. Qoena									
Dishna	7.77	0.34	0.009	1.0	0.18	5.6	11	S,P	0.1
Qus	7.80	0.29	0.008	0.88	0.12	7.33	35	S,P	0.9
Luxor									
Nile-East	7.83	0.22	0.006	1.09	0.14	7.78	25	L,W	0.1
Nile-West	7.86	0.26	0.007	0.80	0.13	6.15	18	L,W	1.2
Armant	7.65	0.70	0.019	0.84	0.11	7.64	16	S,P	8.2
Isna	8.25	0.21	0.006	0.88	0.14	6.29	16	S,P	0.2
Matoana	7.73	0.24	0.006	0.95	0.15	6.33	90	S,P	24.0
10. Aswan									
Kom-ombo	7.64	0.42	0.011	1.16	0.25	4.64	10	L,P	0.8
Aswan	7.94	0.20	0.005	0.83	0.15	5.53	12	L,P	1.2

Governorates and Location	Soil analysis						Nodule characteristics		MPN $\times 10^3$ g^{-1} soil
	PH [1]	EC [2] $dS.m^{-1}$	TSS [3] %	Organic C%	Total N%	C/N ratio	Nodule. [4] plant^{-1}	Size & colour [5]	
Chickpea									
1. El-Kalioubia									
El-Kanater	7.52	0.31	0.008	1.00	0.11	10.00	36	L,P	17.0
2. Assiut									
Abu-Teig	7.31	0.42	0.011	1.06	0.12	8.83	48	L,P	140.0
Pea									
1. El-Behira									
South-Tahrir	7.92	0.29	0.008	0.91	0.17	5.35	11	S,W	0.8
El-Nouhariya	7.81	0.20	0.005	0.72	0.18	4.00	10	S,W	0.9
2. El-Menofia									
Ashmon	7.73	0.40	0.011	1.32	0.28	4.71	31	L,P	17.0

(1) 1: 2.5 soil water suspension. (2) 1: 5 soil water extract. (3)TSS (Total soluble salts) EC × 0.064. (4) Mean of 5 chickpea or pea plants. (5) L= Large (>4 mmØ), S= Small (<3 mmØ), P= Pink and W = White.

Tolerance towards certain pesticides

Two herbicides (Egran 80 %, Cotran 50 %, Cibageigy Comp., Swetherland) and two fungicides (Vitavax syrup 75%, Uny Royal Comp., and Dithane M45 80%, Rohme & Hass Comp.,) were tested for their effect on growth of the rhizobial isolates according to Josey *et al.*, (1979). YMA medium supplemented with various volumes of the pesticide solutions were used to obtain final concentrations of 50,100,250,500.750 and 1000 mg. L^{-1}. active ingredients (a.i.) for each. The prepared pour plates were inoculated with 3-day old *Rhizobium* cultures using metal replicator method. After incubation at 28°C for 48 hrs, rhizobial growth was scored by visual inspection and compared with the corresponding growth on the pesticide-free medium (control). Two plates were prepared for each concentration.

The four pesticides are getting from Pesticides Department, Ministry of Agriculture and Soil Reclamation, Egypt.

Results and Discussion

Characterization of resident rhizobia in Egyptian soils

All soil samples to a depth of 30 cm contained low to moderate levels of salinity, electric conductivity (EC) ranged from 0.17 to 0.70 $dS.m^{-1}$ and had a pH values on the alkaline side being from 7.19- 8.25. Relatively low N content was invariably found (0.10- 0.29%) as well as low organic carbon (0.72-1.41%).

Narrow C/N ratios were accordingly observed in all soil samples (Table 1). In general, all of the aforementioned properties of the investigated soils are within the normal ranges previously recorded for the fertile soils in Egypt (Abd-el-Malek, 1971 and Zahra *et al.*, 1990).

With respect to the characterization of nodules found on the roots of the standing legumes (clover, chickpea and pea), it is clear that plants from different locations invariably harbored nodules with an obvious variation in nodule characteristics and numbers. Most of clover plants were reasonably well modulated since an average number of nodules between 10-102.$plant^{-1}$ were recorded. In only 9 sites, growing clover was poorly nodulated (<20 nodule.$plant^{-1}$). Concerning nodule size and color, it was observed that nodules developed on plants at 18 locations were small > 2 mm diameter) with pink and white color. Large nodules (>3 mm diameter) could be detected on the roots of plants grown in the remaining 8 locations (Table, 1).

Chickpea plants developed average numbers between 36 to 48 nodules $plant^{-1}$ and characterized by their large size and pink color. Pea plants of two sites harbored low number of nodules around 10/plant, all were small and white, while plants from the third site had an average number of 31 large pink nodules.

The total viable count of rhizobia in soil estimated by the plant infection test (MPN) indicated that *R. leguminosarum* bv. *trifolii* were present in high numbers exceeding 10^4 cell g^{-1} in many of the examined fields. This can be attributed to clover, as the main forage crop, is frequently introduced in the crop rotation of most cultivated areas in Egypt. Chickpea as well as pea, on the other hand, are not consistently introduced into the crop rotation and they are usually

cultivated in limited scattered areas. Several studies concerning native rhizobia belonging to various cross-inoculation groups were found to prevail in different soils allover the world in a similar wide range of population density (Moawad & Beck, 1991 and Badr El-Din & Moawad, 1998).

Tolerance towards some pesticides

Out of the 81 *Rhizobium* cultures obtained from clover nodules, 14 isolates (17.3%) were very sensitive to Cotran, even when applied at a low concentration of 50 mg a.i.L^{-1} On the other hand, 61 isolates (75.3%) could tolerate up to 1000 mg L^{-1} Cotran. Similar results were recorded for the effect of Egran on clover *Rhizobium*, where 11 isolates (13.6%) did not grow in presence of the tested concentrations. On the other hand, while 2 isolates (2.5%) tolerated only 50 mg L^{-1} and the remaining 68 isolates (83.95%) could tolerate up to 1000 mg L^{-1} (Table 2). These data are in accordance with those obtained by Martenssen (1992) who stated that, sensitivity of *R. leguminosarium* bv. *trifolii* to the pesticides varied, none of the bacteria were tolerant to all chemical, but rhizobia able to multiply at concentration equal to or higher than recommended field application rates. With respect to the effect of the two fungicides Dithane M45. and Vitavax, clover *Rhizobium* could grow in presence of highest concentrations of Dithane, where 71 isolates (87.7%) developed on YMA medium, provided with 1000mg a.i.L^{-1}. Sensitive isolates, on the other hand, only 8 isolates representing 9.9% of the total number could not tolerate even 50 mg a.i..L^{-1}. The remaining two isolates (2.5%) tolerated 50 mg a.i..L^{-1}. The other fungicide Vitavax, proved to be more inhibitive to clover *Rhizobium*. Only 5 and 2 isolates (6.2 and 2.5%) tolerated the 1000 and 500 mg a.i.L^{-1} concentrations respectively, while 21 and 36 isolates (25.9 and 44.4%) respectively, grew in presence of 50 and 100 mg a.i.L^{-1} of these compound. Seventeen cultures representing 21% of the total isolates failed to grow in presence of any tested concentrations. In this respect, Ruir-Sainr *et al.*, (1984) concluded that the fungicide captafol proved toxic to *R. trifolii* at >75 ug/ml and adversely affected growth at lower concentration. On the other hand, all of the 21 and 29 cultures isolates from chickpea and pea plant nodules respectively, could tolerate the maximum tested concentration of Cotran, Egran or Dithane and Vitavax proved to be less suppressive for these rhizobia. The majority of chickpea *Rhizobium* tolerated relatively low concentrations of the compound, where 9 and 8 isolates (42.9 and 38.1%) could grew in presence of 50 and 100mg a.i.L^{-1}, respectively. Only one isolate tolerated 250 and two isolates tolerated 500 mg a.i..L^{-1}. The fungicides Benomyl (as benlate) or Carbendazim (as bavistin) showed better control of *Fusarium soloni* on chickpea roots than when used separately and developed the maximum number of nodules/plant (Siddiqui *et al.*, 1998).

Out of 29 pea *Rhizobium* isolates, 14 cultures (48.3%) resisted the presence of 1000 mg a.i.L^{-1}. Ten isolates (34.5%) were very sensitive to the pesticides and failed to grow at 50 mg a.i.L^{-1}. The other 5 isolates tolerated between 50 and 500 mg a.i.L^{-1} (Table 2). In this respect, Singh & Wright (1999) found that, all pre-emergence herbicides decreased nodulation and N content of peas; the decreased growth of herbicides-treated plants was due to direct effects of the herbicides on plant and not due to indirect effects on rhizobia. On the other hand, Rensburg & Strijdom(1984) concluded that toxicity of herbicides to rhizobia in vitro did not

necessarily correlate with its effect on nodulation and some are considered suitable for field application. The adverse effect of fungicides on pea *Rhizobium* was also found by Martyniuk *et al.*, (1999) who concluded that, the number of rhizobial cells on pea seeds analyzed within the first hour following inoculant application sharply decreased in case of Dithane compared to the control (no chemical dressing). They also added that Dithane was the most suppressive fungicide compared to Marshal and Sarfun. It is worth to mention that variation between rhizobial isolates to tolerate pesticides gives an opportunity for selecting appropriate strains for using as seed inoculants.

TABLE 2. Growth of rhizobia on YMA amended with different pesticides.

Location	Total R. isolates	Herbicides		Fungicides	
		Cotran	Egran	Dithane	Vitavax
			Clover		
1. El-Behira Abu-Hummus	8	*(1000)8	*(1000)8	*(1000)8	*(50)5,(100)3
Damanhur	5	(1000)5	(1000)5	(1000)5	(50)2,(100)1
Itayai el-Barud	8	(1000)7	(1000)7	(1000)7	(50)1,(100)5
2. El-Gharbia Kafr El-Zaiyat	8	(1000)8	(1000)8	(1000)8	(50)1,(100)7
Tanta	8	(1000)8	(1000)8	(1000)8	(100)8
3. El-Monofia Birkt-el-Sab	4	(1000)4	(1000)4	(1000)4	(100)4
Quweisna	4	(1000)4	(1000)4	(1000)4	(100)3
4. El-Kaliobia Benha	6	(1000)6	(1000)6	(1000)6	(50)2,(100)4
5. Giza Tanash	2	(250)1, (1000)1	(1000)1	(1000)1	(1000)1
El-Aiyat	7	(1000)7	(1000)7	(1000)7	(50)5,(100)1, (500)1
El-Saff	1	–	(50)1	–	–
6. El-Menia Deir-Mowas	1	–	(1000)1	(1000)1	(50)1
7. Assiut Sedfa	2	(100)1, (500)1	(1000)1	(1000)1	–
8. Sohag Tahta	1	(50)1	(1000)1	(1000)1	(50)1
Sohag	1	–	–	–	–
Girga	2	(100)1	(50)1,(1000)1	(1000)2	(50)1,(1000)1
El-Balyana	2				
9. Quena Dishna	1	–	–	–	–
Qus	2	(100)1	(1000)1	(1000)2	
Luxor Nile-East	1	–	–	(1000)1	(50)1
Nile-West	1	–	–	(50)1	–
Armant	1	–	–	–	–
Isna	1	(1000)1	(1000)1	(1000)1	(500)1
Matanna	1	–	–	–	–
10. Aswan Kom-ombo	1	(1000)1	(1000)1	(1000)1	(1000)1
Aswan	2	–	–	–	–

TABLE 2. Contd.

Location	Total R. isolates	Herbicides		Fungicides	
		Cotran	Egran	Dithane	Vitavax
Chickpea					
1. El-Kaliobia El-Kanater	20	(1000)20	(1000)20	(1000)20	(50)9,(100)8, (250)1,(500)2
2. Assiut Abu-Teig	1	(1000)1	(1000)1	(1000)1	(50)1
Pea					
1.El-Behira South-Tahrir	15	(1000)15	(1000)15	(1000)15	(50)1,(100)1,(1 000)12
El-Noubariya	6	(1000)6	(1000)6	(1000)6	(250)2,(1000)1
2. El-Monofia Ashmun	8	(1000)8	(1000)8	(1000)8	–

*(pesticides concentration) isolates number

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دراسات بيئية على الريزوبيا المتوطنة بالأراضي المصرية
٢- أثر مبيدات الحشائش والمبيدات الفطرية على نمو الريزوبيا

حسين عبد المقصود – إسماعيل حسنى وفاطمة حلمى عبد الظاهر.

قسم الميكروبيولوجيا بالمركز القومى للبحوث و*قسم الميكروبيولوجيا بكلية الزراعة جامعة القاهرة.

حيث أن زراعة البقوليات فى الأراضي القديمة تعتمد بالدرجة الاولى على الريزوبيا المتوطنة بها ، فأنه يصبح من الأهمية بمكان التعرف على مدى تواجد وانتشار هذه البكتريا فى مختلف أنوع الأراضي. أجريت هذه الدراسة لإلقاء المزيد من الضوء على مدى حساسية الريزوبيا المتوطنة لبعض المعاملات بالمبيدات الفطرية ومبيدات الحشائش.

تناولت الدراسة تجميع عينات من التربة من أراضى مزروعة بمحاصيل البرسيم ، الحمص والبسلة ، شملت ٣١ موقعا فى عشرة محافظات فى الدلتا والوجه القبلى. تم دراسة خواص التربة ، كما تم تقدير العدد الاحتمالي للريزوبيا لكل محصول على حدة ودراسة خواص العقد الجذرية المتكونة. وكذلك تم عزل ١٣١ مزرعة من الريزوبيا للمحاصيل الثلاث (٨١ من البرسيم ،٢١ من الحمص و٢٩ من البسلة).

اختبرت جميع عزلات الريزوبيا لمدى تحملها لتركيزات مختلفة من مبيدات الحشائش Cotran, Egran ومبيدات الفطريات Dithane, Vitavax. وقد أظهرت النتائج أن نسبة ١٧,٣٪ من ريزوبيا البرسيم كانت حساسة تجاه مبيد Cotran بينما تحملت ٧٥,٣٪ من العزلات تركيزات حتى ١٠٠٠ مجم مادة فعالة/لتر من المبيد. أما النسبة الباقية (٧,٤ ٪) فقد استجابت بدرجات متفاوته تجاه هذا المبيد. وقد كان التأثير لمبيد الحشائش Egran أقل لنفس التركيزات تجاه نفس الريزوبيا. أما بالنسبة للمبيدات الفطرية تحت الدراسة فكان لها أثرا مثبطا بدرجة اكبر على الريزوبيا من مبيدات الحشائش ، حيث كان للمبيد الفطرى Vitavax تأثير مثبط واضح على ريزوبيا البرسيم فلم يستدل الا على عزلتين أمكنهما تحمل تركيز ٥٠٠ مجم / لتر ، بينما لم تتمكن ١٧ عزلة أخرى من النمو على التركيز الأقل من المبيد (٥٠مجم / لتر) .

هذا وقد تمكنت جميع عزلات الحمص و البسلة من تحمل التركيز الأعلى (١٠٠٠ مجم/ لتر) من مبيدات Cotran, Egran and Dithane بينما كان للمبيد الفطرى Vitavax تأثير مثبط واضح عليها.

ومن الناحية التطبيقية فأن هذه الدراسة تلقى مزيدا من الضوء على الأثر الضار للمبيدات عامة- سواء كانت فطرية أو مبيدات حشائش- على ميكروب الريزوبيا الفاعل فى تثبيت النيتروجين الجوى بالمعاشرة مع البقوليات .