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Full Length Research Paper

Fungicide, antibiotic, heavy metal resistance and salt tolerance of root nodule isolates from *Vicia palaestina*

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The objective of this study was to investigate the effects of fungicides, antibiotics, heavy metal and salt on growth of *Rhizobium* isolates which isolated from the *Vicia palaestina* from Şanlıurfa, Turkey. Twenty *Rhizobium* bacteria were isolated. Isolates were tested for their tolerance to mancozeb, carbendazim or mancozeb + carbendazim. The effect of the fungicides on the isolates of *Rhizobium* was variable, depending on the fungicide and isolate. All of the rhizobial isolates showed resistance to the antibiotic (µg ml^{-1}); streptomycin sulphate (75) and to heavy metals Cu (0.5 mmol), Cd (0.065 mmol), Zn (0.125 and 0.250 mmol) and Mn (0.75 mmol). An experiment was conducted to find out the effects of varying salt concentrations ranging from 1 to 4 % NaCl. Isolates (V1, V9, V13, V14, V15, V18 and V20) were found to tolerate a relatively high salt concentration.

Key words: *Rhizobium*, *Vicia palaestina*, fungicide, antibiotic, heavy metal and salinity.

INTRODUCTION

The pastures are important part of agricultural systems and to increase their productivity and quality, resistant forage legumes are needed (Albayrak and Sevimay, 2005). *Vicia* species are commonly grown to provide seed and hay in many different farming systems in Turkey (Avcıoğlu et al., 2009). *Vicia* species are the primary legumes used in pasture in Şanliurfa, Turkey (Cevheri and Polat, 2009). *Vicia* species have a symbiotic life with specific bacteria called *Rhizobium* (Hansen, 1994).

Rhizobium bacteria are genetically diverse and physiologically heterogeneous group of symbiotic nitrogen fixing bacteria that forms nodules in the roots within the bacteria which fix atmospheric nitrogen into ammonia (Alexander, 1984). A fully functional symbiosis requires successful survival ability of bacteria even under adverse environmental conditions (Herridge et al., 2008). Within the soil, *Rhizobium* bacteria encounter various stresses that affect their growth, their initial steps of symbiosis and the capability of nitrogen fixation (Ramadoss and Sivaprakasam, 1991).

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Using fungicides for crop diseases control in legume fields has contributed to increasing yield and improved quality (Fox et al., 2007). Fungicides not only affect plant growth but also have a detrimental effect on soil microorganisms growth and metabolism (Castro et al., 1997; Malik and Tesfai, 1983; Lennox and Alexander, 1981). Some studies have been evaluated on the effect of different fungicides on *Rhizobium* bacteria growth and nitrogen fixation capacity (Rennie and Dubetz, 1984; Tu, 1982, 1981). *Rhizobium* isolates have different wide resistance to fungicides (Diğrak and Kirbağ, 1996; Martyniuk et al., 1999a; 1999b), antibiotics (Alexander et al., 2006; Hungria et al., 2001) and salinity (Cardovilla et al., 1994, 1999; Ködöböcz et al., 2009).

The objective of this study is to investigate the effects of fungicides, antibiotics, heavy metal and salt on growth of *Rhizobium* isolates.

MATERIALS AND METHODS

Twenty *Rhizobium* bacteria were isolated by standard method (Jordan, 1984) from nodules of *Vicia palaestina* in Şanliurfa, Turkey. In all cases, large sized randomly chosen active (pink coloured) nodules were selected and surface sterilised with 3% hypochlorite solution followed by rinsing five times with sterile distilled water. Nodules were dissected with a sterile scalped and

was transferred onto yeast extract mannitol agar (YEMA) slants with an inoculation loop (Jordan, 1984). Isolates were purified and tested for gram reaction, colony structure mucoidly and catalase (Hungria et al., 2001; Jordan, 1984).

Determination of fungicide resistance

Two fungicides commonly used on crops: Mancozeb (80% active ingredient in granular formation) and carbendazim were tested for their inhibitory effect on the colonisation of rhizobial isolates. Macozeb and carbendazim were applied at recommended doses of 200 g 100 l^{-1} and 300 g 100 kg^{-1} seed, respectively. Concentrations of these fungicides were added to yeast exract broth and were inoculated with 80 µl of the test culture (10^8 cell/ml) and incubated at 28°C for 72 h. Tolerance of the isolates to fungicides was studied by the method described in Kale et al. (1989). Controls without the inclusion of fungicides were also maintianed simultaneously. Serial dilutions of cultures were prepared and colonies were obtained on YEMA (0.2 g, $MgSO_4$ $7H_2O$; 0.5 g, KH_2PO_4; 0.1 g, NaCl; 10 g mannitol; 0.5 g yeast extract; 15 g, agar; 1000 ml, distilled water) (Jordan, 1984).

Determination of heavy metals resistance

A total of 20 *Rhizobium* isolates of *V. palaestina* was assessed for their resistance against four different heavy metals (Cu, Cd, zinc sulphate (Zn) and Mn) using 5 levels of concentrations. The resistance of isolates to heavy metals was evaluated on solid YEMA medium. The stock solutions of heavy metals (mmol) were filtered, sterilized and added to sterile agar as follows: $CuCl_2.2H_2O$ 0.5, 1.0, 1.5, 2.0 and 2.5; $CdCl_2$ 0.065, 0.125, 0.250, 0.500 and 1.0; $ZnSO_4.7H_2O$ 0.125, 0.250, 0.500, 1.0 and 2.0; $MnSO_4.4H_2O$ 0.75, 1.5, 3.0, 6.0 and 12. The plates were inoculated with about 10^8 cells and the bacterial growth was evaluated after 7 days at 28°C (Ausili et al., 2002; Chaudri et al., 1992a). Isolates were considered resistant when growth occured and sensitive when no growth was detected.

Determination of antibiotic resistance

Resistance to antibiotics was evaluated on YEMA for the isolated rhizobial strains. Fresh solutions of filtered (0.22 µm) sterilized antibiotics were added to melted YEMA medium to give the following concentrations (µg ml^{-1}): Chloromphenicol, 20; streptomycin sulphate, 75; ampicillin, 10; erytromycin, 50; penicilin G, 10; rifampicin, 30 (Hungria et al., 2001; Sambrook et al., 1989). Each isolate was replicated twice per antibiotic concentration, by dispensing 10 µl (10^8 cell/ml) per Petri plate. Plates were incubated at 28°C and scored after 72 h. Isolates were considered resistant when growth occured and sensitive when no growth was detected.

Determination of NaCl tolerance

Resistance of the isolates against different concentrations of NaCl was determined on minimal salt medium containing 1.5% w/v agar, 20 mM HEPES (N-2-hydroxyethane-sulphonic acid) and 20 mM mannitol with NaCl in varying concentrations ranging from 1, 2, 3 and 4% (w/v) (Marsudi et al., 1999). The plates contained 25 ml of the medium and a loopfull of each isolate was streaked on Petri dishes (Hungria et al., 2001).

The plates were incubated for 72 h at 28°C and the susceptibility to NaCl was recorded as a positive or negative result.

Statistical analysis

Data were analysed for statistical significance using the analysis of variance package (ANOVA) using Microsoft Excell (MS) 98.

RESULTS AND DISCUSSION

Nitrogen fixation by root nodulating isolate of *Rhizobium* contributes a significant input into the many types of farming systems. For instance, even 80% of total N in pasture legume plants can be supplied by root nodule bacteria (Goring and Laskowski, 1982). Fungicides applied as seed dressings protect germinating seeds and young seedlings against fungal pathogens and pests (Martyniuk et al., 1999b). In the case of leguminous plants, treatment of seeds with *Rhizobium* inoculants is also very important (Martyniuk et al., 2002). When *Rhizobium* bacteria are inoculated on chemically treated seeds of crop, their survival and capacity to induce symbiosis can be markedly reduced due to possible toxic effects of fungicides on these bacteria (Rennie and Dubetz, 1984).

Therefore, the effect of fungicides on legume nitrogen fixation is of great importance. Fungicides may inhibit nodulation by affecting cellulolytic and pectolytic enzyme production by the *Rhizobium* (Hansen, 1994). These enzymes secreted by *Rhizobium* is essential for root hair penetration (Hansen, 1994). Many studies have indicated differential effect of fungicides on *Rhizobium*, nodulation and nitrogen fixation (Fox et al., 2007; Ramadoss and Sivaprakasam 1991; Rennie and Dubetz, 1984). In the present study, all the isolates of *Rhizobium* were tested for their tolerance to mancozeb, carbendazim or mancozeb + carbendazim.

The results of the study were statistically analysed as given in Table 1. The isolates, fungicides and isolates x fungicides interaction mean squares are significant at 1% level. The effect of the fungicides on the isolates of *Rhizobium* was variable, depending on the fungicide and isolate (Table 1). All isolates in the presence of the tested fungicides applied at field dose showed growth inhibition. Addition of mancozeb and also carbendazim was found to decrease growth for *Rhizobium* isolates at concentration recommended for field application. Mancozeb of 98% inhibited the isolate V5 (Table 1). Inhibitory effects of mancozeb on the growth of *Bradyrhizobium japonicum* and *Rhizobium* sp. were suggested by Martyniuk et al. (2002) and Ramadoss and Sivaprakasam (1991). Carbendazim of 94% inhibited the V3, V9 and V10 isolate. Carbendazim + mancozeb mix of 97% inhibited the V5 isolate (Table 1).

All the isolates of *Rhizobium* sp. were tested for their resistance to the following heavy metals Cu, Cd, Zn or Mn. 100 % of the isolates resistant to Cu (0.5 mmol), Cd (0.065 mmol), Zn (0.125 mmol and 0.250 mmol) and Mn (0.75 mmol) (Table 2). Manganase was the most heavy metal resistant (65% at 1.5 mmol) followed by Cu resistant (90 % at 1.0 mmol), zinc resistant (15%) and cadmium resistant (0%) at 0.5 mmol. Cadmium resistant the least (25 %) at 0.125 mmol and three levels of con-

Table 1. Effect of fungicides on the growth of rhizobia in yeast extract mannitol agar (10^6 cell ml^{-1}).

Isolate	Fungicides			
	Carbendazim	Mancozeb	Carbendazim+mancozeb	Control
V1	14 ± 0.03#	7 ± 0.01	11 ± 0.02	150 ± 0.02
V2	21 ± 0.01	9 ± 0.01	15 ± 0.02	286 ± 0.02
V3	24 ± 0.01	10 ± 0.01	19 ± 0.01	451 ± 0.03
V4	23 ± 0.01	9 ± 0.01	12 ± 0.02	213 ± 0.01
V5	34 ± 0.01	5 ± 0.01	6 ± 0.02	280 ± 0.04
V6	51 ± 0.01	8 ± 0.01	19 ± 0.01	248 ± 0.04
V7	31 ± 0.03	9 ± 0.01	21 ± 0.01	313 ± 0.06
V8	61 ± 0.01	12 ± 0.02	17 ± 0.02	296 ± 0.02
V9	14 ± 0.01	7 ± 0.01	16 ± 0.03	275 ± 0.01
V10	11 ± 0.02	6 ± 0.02	11 ± 0.02	194 ± 0.01
V11	13 ± 0.02	3 ± 0.03	7 ± 0.01	136 ± 0.02
V12	12 ± 0.01	13 ± 0.02	11 ± 0.01	195 ± 0.03
V13	21 ± 0.02	21 ± 0.03	14 ± 0.01	197 ± 0.01
V14	24 ± 0.01	3 ± 0.03	10 ± 0.04	125 ± 0.01
V15	19 ± 0.02	4 ± 0.02	12 ± 0.02	129 ± 0.03
V16	30 ± 0.02	3 ± 0.02	9 ± 0.01	115 ± 0.02
V17	37 ± 0.03	11 ± 0.01	13 ± 0.01	238 ± 0.01
V18	51 ± 0.01	10 ± 0.01	13 ± 0.05	210 ± 0.02
V19	28 ± 0.01	11 ± 0.02	15 ± 0.01	261 ± 0.02
V20	40 ± 0.01	14 ± 0.01	21 ± 0.02	285 ± 0.01

ANOVA	df	Mean square
Replication	1	18.2
Fungicides	3	222957.7**
Control and others	1	668714.7**
Others	2	79.2**
Isolates	19	1861.5**
Isolates x fungicides	57	1284.4**
Error	79	635.1

**Significant at the 1% probability level; #standard deviation.

centration were lethal effect. Altough heavy metal is usually not a problem in Şanlıurfa soils, Turkey, Cu and Zn concentrations of 1 mmol and 0.250 mmol, respectively were tolerated by the *Rhizobium* isolates (Table 2). Our results suggest that Cu resistance may result from a sequestration or binding mechanism. Polysaccharides or proteins are potential binding sites (Cervantes et al., 1994). Cu^{2+} is accumulated in the periplasm of *Pseudomonas* by periplasmic CopA and CopB proteins (Silver and Phung, 1996). The ability to resist the tested heavy metals decreased with increase in concentration. The high con-centration was more suppressive to the bacterial growth. Isolates could resist all heavy metals when applied in low concentration (100%), but at high concentration, isolates are adversely affected. No growth was obtained at high concentration of Zn, Mn and Cd, whereas only 5% of isolates could survive at high concentration of Cu.

Antibiotic sensitivity behaviour of *Rhizobium* sp. isolates presented in Table 3 revealed that all the isolates were resistance to one or more antibiotics. The two isolates (V13 and V15) were resistant to chloramphenicol, while they were sensitive to rifampicin (Table 3). Resis-tance to chloramphenicol was also reported by Xavier et al. (1998), for some cowpea *Rhizobium* from Brazil soils. A plasmid of approximately 70 kb was reported to be responsible for resistance to metal in *Rhizobium* isolates by Lakzian et al. (2002). In this study, all the isolates were resistant to streptomycin sulphate but sensitive to rifampicin. Similar observations were recorded by Marsudi et al. (1999) and Ködöböcz et al. (2009) who found that the growth of *Rhizobium* was very slow on rifampicin. The resistance of *V. palaestina* rhizobia to antibiotics showed that all the tested isolates (100%) exhibited resistance to streptomycin, 45 and 35% to penicillin G and erytromycin, only 10% to chloramphe-

Table 2. Effect of different concentrations of Cu, Cd, Zn and Mn on the growth of *Rhizobium* sp. İsolates.

Metals	Concentration (mmol)	No. of resistant isolates (%)
Cu	0.5	100
	1.0	90
	1.5	40
	2.0	25
	2.5	5
Cd	0.065	100
	0.125	25
	0.250	0
	0.500	0
	1.0	0
Zn	0.125	100
	0.250	100
	0.500	55
	1.0	15
	2.0	0
Mn	0.75	100
	1.5	65
	3.0	20
	6.0	0
	12.0	0

Total number of isolates: 20.

nicol. In the presence of ampicilin, the isolates were intermediate (20%) and sensitive (80%). Growth of these isolates was fully inhibited by streptomycin. The 6 antibiotics could be arranged in descending order as follows: Streptomycin > penicillin G > erytromycin > chloramphenicol > ampicillin > rifampicin. The tested rhizobial isolates can be grouped into at least 11 groups; these group of isolates are for example A. V1 and V2; resistant to erytromycin and streptomycin sulphate, sensitive to chloramphenicol, penicillin G and rifampicin, intermediate to ampicillin; B. V3, V16 and V17 similar to A, but sensitive to ampicillin; C. V4 and V5; intermediate to erytromycin, resistant to streptomycin sulphate, sensitive to chloramphenicol, penicillin G, ampicillin and rifampicin; D. V6 and V7; sensitive to erytromycin, chloramphenicol, ampicillin and rifampicin, resistant to streptomycin sulphate, intermediate to penicillin G; E. V8, V9 and V10, similar to D but resistant to penicilin G; F. V11 and V12; similar to C, but resistant to penicillin G; G. V13 similar to F, but resistant to chloramphenicol; H. V14 similar to F, but sensitive to chloramphenicol; I. V18 resistant to erytromycin, streptomycin sulphate and penicillin G, sensitive to chloramphenicol and rifampicilin; J. V19 intermediate to erytromycin, chloramphenicol and ampicillin, resistant to streptomycin sulphate and penicillin G, sensitive to rifampicin; K. V20; intermediate to erytromycin, sensitive to chloramphenicol, ampicillin and rifampicin, resistant to streptomycin sulphate and penicillin G (Table 3).

In this study, *V. palaestina* isolates showed a wide range of behavior with regard to antibiotics and heavy metals. All the streptomycin sulphate were resistant to Zn concentrations of 0.125 and 0.250 mmol, Cu concentration of 0.5 mmol and Cd concentration of 0.065 mmol (Tables 2 and 3). In genetic studies, these heavy metal and antibiotic resistance traits should be extremely valuable as positive selection markers. Further experiments are being carried out to identify heavy metal and antibiotic resistant genes in rhizobial isolates.

It is known that salt stress significantly reduces nitrogen fixation in legume (Singleton et al., 1982). To date, only some rhizobial isolates have been shown to grow under high saltly soils. Several isolates have been reported to grow at high salt concentrations (>3%) (Hungria et al., 2001; Singleton et al., 1982). In our study, the effects of salt concentrations were variable, depending on the salt concentrations and isolates (Table 4). Differences in NaCl tolerance were observed among the isolates, 100% grew at 1 and 2% NaCl, 65% isolates grew at 3% NaCl and 40% isolates grew at 4% NaCl (Table 4). Similar results were obtained with a *Leuceana Rhizobium* (Hashem et al., 1998). Salt tolerance is an important phenotype under investigation. Tolerance towards high salt concentrations has been reported in several tropical *Rhizobium* isolates, isolated from different arid or saline sites, that have been shown to be efficient inoculants (Elsheikh and Wood, 1989; Zou et al., 1995). These results agree well with those of Martinez-Romero et al.

Table 3. Effect of tested antibiotics on the growth of *Rhizobium* sp.

Isolate	Antibiotic sensitivity					
	E50	S75	C20	P10	A10	R30
V1	R	R	S	S	I	S
V2	R	R	S	S	I	S
V3	R	R	S	S	S	S
V4	I	R	S	S	S	S
V5	I	R	S	S	S	S
V6	S	R	S	I	S	S
V7	S	R	S	I	S	S
V8	S	R	S	R	S	S
V9	S	R	S	R	S	S
V10	S	R	S	R	S	S
V11	I	R	S	R	S	S
V12	I	R	S	R	S	S
V13	I	R	R	R	S	S
V14	I	R	S	S	S	S
V15	R	R	R	S	S	S
V16	R	R	S	S	S	S
V17	R	R	S	S	S	S
V18	R	R	S	R	I	S
V19	I	R	I	R	I	S
V20	I	R	S	R	S	S

R, Resistant to antibiotic; I, intermediate to antibiotic; S, sensivite to antibiotic. E50 erytromycin, S75 streptomycin sulphate, C20 chloramphenicol, P10 penicillin G, R30 rifampicin, A10 ampicillin.

Table 4. Effect of NaCl concentration on *Rhizobium* sp.

Isolate	NaCl concentration (%)			
	1	2	3	4
V1	+*	+	+	+
V2	+	+	+	-
V3	+	+	+	-
V4	+	+	-	-
V5	+	+	+	-
V6	+	+	-	-
V7	+	+	-	-
V8	+	+	-	-
V9	+	+	+	+
V10	+	+	-	-
V11	+	+	+	-
V12	+	+	+	-
V13	+	+	+	+
V14	+	+	+	+
V15	+	+	+	+
V16	+	+	-	-
V17	+	+	+	-
V18	+	+	+	+
V19	+	+	-	+
V20	+	+	+	+

+*, Growth; - no growth.

(1991), who reported that same isolates *Rhizobium* species were NaCl tolerant, studies on the genetic basis of tolerance to antibiotics, metals and NaCl suggest a chromosomal rather than a plasmid location of genes. The isolates can be divided into three groups, the first group of seven isolates (V1, V9, V13, V14, V15, V18 and V20) were able to grow on all tested NaCl concentrations (Table 4), the second group of seven isolates (V2, V3, V5, V11, V12, V17 and V19) were able to grow on three tested NaCl concentrations and the last group of six isolates (V4, V6, V7, V8, V10 and V16) were able to grow on two tested NaCl concentrations. The first group is the most resistant, whereas the last is the susceptible group. In this study, a clear distiction form was expressed between fungicide, antibiotic and heavy metal resistance, salt tolerance and rhizobial isolates.

The local isolates used in this study might be different in characterization than other *Vicia* isolates. Further studies are needed to study the genes involved in heavy metal, antibiotic resistance and the relationship between these genes and the symbiotic genes. These isolates can be assessed for their symbiotic efficiency and suitability as inoculant for cultivated edible *V. palaestina*.

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