African Journal of Agricultural Research Vol. 7(10), pp. 1462-1467, 12 March, 2012
Available online at http://www.academicjournals.org/AJAR
DOI: 10.5897/AJAR11.061
ISSN 1991-637X © 2012 Academic Journals

Full Length Research Paper

Tolerance of rhizobia isolated from *Trifolium* species in Southeast region, Şanliurfa, Turkey

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Accepted 13 September, 2011

The objective of this study was to investigate the effects of salt, fungicide, herbicide, antibiotics, heavy metal on growth of *Rhizobium* isolates from root nodules of *Trifolium* species from Şanliurfa, Turkey. All of the isolates, expect TC2 were found to tolerate a relatively high salt concentration. None of the isolates grew at pH 4 and four isolates grew at pH 4.5. All of the rhizobial isolates showed resistance to streptomycin sulphate, chloramphenicol, ampicillin, penicillin G and kanamycin and to Zn (0.1 g L^{-1}). T4 isolate showed sensitivity to penicillin G and kanamycin. Isolates were tested for their tolerance to fungicide or herbicide. The effect of the pesticides on the rhizobial isolates was variable, depending on the fungicide or herbicide and the isolate.

Key words: *Trifolium* species, isolate, NaCl, antibiotics, pH, heavy metal, pesticide.

INTRODUCTION

Root nodulating bacteria are particularly useful due to their symbiotic nitrogen fixing ability in symbiosis with legume plants. *Trifolium* species are found in the Southeast region of Turkey. The region is characterized by semi-arid climates. The leguminous plants growing here are used as foliage and green manure (Cevheri and Polat, 2009). The naturally growing leguminous plants in arid and semi arid regions are subjected to severe environmental conditions (Zahran, 1999; Watkinn et al., 2003). Among several environmental conditions such as temperature extremes, pH stress, salinity, nutrient deficiency, heavy metals and chemicals were probably the most problematic (Choudhury et al., 2010; Evans et al., 1980; Ghollarata and Raiesi, 2007; Giller et al., 1993; Herridge et al., 2008). *Rhizobium* bacteria showed marked variation in salt tolerance (Zahran, 1999). *Rhizobium* isolates were grown at salt concentrations of more than 300 mm L^{-1} salt, but some isolates of *Rhizobium* species were inhibited by 100 mmol L^{-1} (Elsheikh and Wood, 1989; Zhang et al., 1991).

Before understanding field application of promising inoculants, identification of certain markers (e.g., heavy metal, pH, antibiotic resistance) is necessary for ecological competitiveness (Ausili et al., 2002; Dourin et al., 1996; Evans et al., 1980; Zahran, 1999). Several studies have demonstrated that *Rhizobium* species can tolerate many fungicides (Allievi and Gigliotti, 2001; Castro et al., 1997; Lennox and Alexander, 1981; Milosevic and Govedarica, 2002; Ramadoss and Sivaprakasam, 1991). The aim of this research was to determine the tolerance of rhizobial isolates of *Trifolium* species at salinity, pH, heavy metal, antibiotics, tested fungicide and herbicide concentrations.

MATERIALS AND METHODS

Sampling site

The sampling site was in the Southeast region (Şanliurfa) of Turkey, a semi-arid zone with a mean rainfall of 458.4.mm $year^{-1}$ and a mean minimum and maximum temperature of -6.8 and 47°C, respectively. The soil is alkaline with a pH of 7.57.

Isolation of rhizobial isolates

For the isolation of bacteria, *Trifolium* species were collected at the beginning of flowering. Bacterial isolates were isolated from nodules using the method of Jordan (1984). In all cases, large sized randomly chosen active (pink coloured) nodules were selected and

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Table 1. Isolates used in this research.

Isolate	Host plant
TT3, TT4, TT5	*Trifolium tomentosum*
Tr3, Tr9	*Trifolium repens*
TC2, TC5, TC7	*Trifolium compestre*
TrM1, TrM2, TrM4, TrM5, TrM6, TrM7	*Trifolium metusum*

Table 2. Tolerance of rhizobial isolates to NaCl.

Isolate	NaCl (%)								
	1	1.5	2	2.5	3	3.5	4	4.5	5
TrM1	+	+	+	+	+	+	+	+	+
TrM4	+	+	+	+	+	+	+	+	+
TrM7	+	+	+	+	+	+	+	+	+
TrM6	+	+	+	+	+	+	+	+	+
TC5	+	+	+	+	+	+	+	+	+
TC7	+	+	+	+	+	+	+	+	+
TC2	+	+	+	+	+	+	+	-	-
TrM2	+	+	+	+	+	+	+	+	+
TT3	+	+	+	+	+	+	+	+	+
TT5	+	+	+	+	+	+	+	+	+
Tr3	+	+	+	+	+	+	+	+	+
TrM5	+	+	+	+	+	+	+	+	+
Tr9	+	+	+	+	+	+	+	+	+
TT4	+	+	+	+	+	+	+	+	+

+, Colonies observed on YEMA medium; -, No growth observed.

surface sterilised with 3% hypochlorite solution followed by rinsing 5 times with sterile distilled water. Nodules were dissected with a sterile scalped and was transferred onto yeast extract mannitol agar (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) slants with an inoculation loop (Jordan, 1984). Isolates were purified and were tested for Gram reaction, colony structure mucoidly and catalase (Hungria et al., 2001). Acid and alkali production was determined in YEMA medium with bromothymol blue indicator (0.0025%) (Hungria et al., 2001). The isolates and plants used were listed in Table 1.

Stress tolerance

Cultures (10 µl) were inoculated into YEMA containing NaCl (w/v) (1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5 and 5%) and by incubating them at 30°C (Hungria et al., 2001). To determine the pH tolerance, the isolates were grown in yeast extract mannitol broth (YEM broth) in which the pH was adjusted to 4, 4.5, 5, 6, 7, 8 and 9 (Rodriguez et al., 2006; Shamseldin and Werner, 2005).

Intrinsic antibiotic and heavy metal resistance were determined on YEMA agar medium containing the following filter sterilized antibiotics or heavy metals; Chloramphenicol (10 mg L^{-1}), Kanamycin (50 mg L^{-1}), Streptomycin sulphate (50 mg L^{-1}), Penicillin G (15 µg ml^{-1}), Ampicilin (10 µg ml^{-1}), $CuCl_2.2H_2O$ (0.1 g L^{-1}), $ZnSO_4.7H_2O$ (0.1 g L^{-1}), $CdCl_2$. $2H_2O$ (20 µg ml^{-1}) and $HgCl_2$ (5 µg ml^{-1}) (Dourin et al., 1996; Zhang et al., 1991). Antibiotics were prepared after Sambrook et al. (1989), sterilised by membrane filtration (0.22 µm) and added to YEMA medium (Hungria et al., 2001).

Effects of fungicide or herbicide on isolates

Fungicide and herbicide commonly used on crops were tested to determine whether any would inhibit recolonization of *Rhizobium* isolates. Two pesticides were used in this investigation. The fungicide Tebicur-2DS is commonly known as tebuconazole with 2% active ingredient (a.i.) fungicide was manufactured by Safa Agriculture, Konya, Turkey. The herbicide was also used: Helga super herbicite (Quizalofop-p-ethyl, ethyl (R)-2-[4(-6-chloro-2-quinoxalinyloxy)-phenoxy-]propionate, Agro Best Group, Turkey). Doses of formulations were followed by recommendations given by the manufacturers. Fungicide was applied at the rate of 75 g 100 L^{-1}, 150 g 100 L^{-1} and 300 g 100 L^{-1}. Herbicide was applied at the rate of 50, 100 and 150 ml L^{-1}.

Concentrations of these pesticides were added in yeast extract mannitol broth (50 ml) and were inoculated with the test culture (10^6 cell ml^{-1}). The flasks were then put in a rotary shaker at 30°C and 120 rev min^{-1} for 72 h. Tolerance of the isolates to fungicides was studied by the method described by Kale et al. (1989). Separate experiments were conducted for each pesticide (that is three different experiments). There were two replicates. Serial dilutions of cultures were prepared and obtained on yeast extract mannitol agar (YEMA). Control teratments containing no fungicide or herbicide were included in each treatment. Data were analysed for statistical significance using the analysis of variance package included in Microsoft Excel 98.

RESULTS AND DISCUSSION

The 14 rhizobial isolates from naturally occuring root nodules of *Trifolium* species growing in Şanliurfa, Turkey were obtained to effects of salinity, different pH values, heavy metal, antibiotics, fungicide and herbicide concentrations. All of the isolates were Gram negative, catalase positive and moderately motile. The effects of salt concentrations on nitrogen fixation have been reported in several studies (Gaballah and Gomaa, 2005; Thrall et al., 2009; Zahran et al., 1999). Salinity stress is one of the most serious factors limiting the productivity of agriculture (Thrall et al., 2009; Zahran, 1999). The growth responses of 14 rhizobial isolates to NaCl at 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5 and 5% were shown in Table 2. Isolate TC2 was not grown in 4.5 and 5% NaCl (Table 2). All of isolates grew in YEM medium with pH values of 5.0 and 9.0 (Table 3), as observed for other root nodule bacteria (Hungria et al., 2001; Liu et al., 2007; Palaniappan et al., 2010).

Table 3. Effect of different pH values on the growth of isolates.

Isolate	pH						
	4	4.5	5	6	7	8	9
TrM1	-	-	++	++	++	+	+
TrM4	-	-	++	++	++	+	+
TrM7	-	-	++	++	++	+	+
TrM6	-	-	+	+	++	+	+
TC5	-	-	++	++	++	+	+
TC7	-	-	+	+	++	+	+
TC2	-	-	+	+	++	+	+
TrM2	-	+	++	++	++	+	+
TT3	-	+	++	++	++	++	+
TT5	-	-	+	+	++	+	+
Tr3	-	+	++	++	++	+	+
TrM5	-	-	+	+	++	+	+
Tr9	-	-	++	++	++	+	+
TT4	-	+	++	++	++	+	+

+, weak growth; ++, good growth; -, no growth.

Table 4. Effects of heavy metals and antibiotics on growth of *Rhizobium* sp. isolates.

Isolate	Heavy metal resistance				Antibiotic resistance				
	$CuCl_2$	$ZnSO_4$	$CdCl_2$	$HgCl_2$	S50	C10	P15	A10	K50
TrM1	S	R	R	S	R	R	R	R	R
TrM4	S	R	R	S	R	R	R	R	R
TrM7	S	R	R	S	R	R	R	R	R
TrM6	S	R	S	S	R	R	R	R	R
TC5	S	R	R	S	R	R	R	R	R
TC7	I	R	S	R	R	R	R	R	R
TC2	S	R	S	R	R	R	R	R	R
TrM2	S	R	S	S	R	R	R	R	R
TT3	I	R	S	S	R	R	R	R	R
TT5	I	R	S	S	R	R	R	R	R
Tr3	I	R	S	S	R	R	R	R	R
TrM5	R	R	S	S	R	S	S	S	R
Tr9	R	R	S	S	R	R	R	R	R
TT4	R	R	S	S	R	R	S	R	S

R, resistant to antibiotic or heavy metal ; I, intermediate to antibiotic or heavy metal; S, sensivite to antibiotic or heavy metal. S50 Streptomycin sulphate, C10 Chloramphenicol, P15 Penicillin G, A10 Ampicillin, K10 Kanamycin.

In our study, the effects of pH values were observed among the isolates, 100% grew at pH 5, 6, 7, 8 and 9; 28.6% isolates grew at 4.5 pH (Table 3). None of the isolates grew at pH 4 (Table 3). The optimum pH for *Rhizobium* isolates growth usually well between 6 and 7 (Jordan, 1984). Although a few rhizobial isolates grew well at a pH less than 5 (Rodriguez et al., 2006), some isolates of *Rhizobium* species grew at pH 12 (Surange et al., 1997). The antibiotic resistance of the rhizobial isolates showed a high a level of resistance against kanamycin, streptomycin sulphate, ampicillin, penicillin G and chloramphenicol (Table 4). TT4 isolate showed sensitivity to penicilin G and kanamycin. TrM5 showed sensitivity to streptomycin sulphate, chloramphenicol and ampicillin (Table 4). Different authors have reported effect of antibiotics on root nodule bacteria (Liu et al., 2007; Zhang et al., 1991; Hungria et al., 2001). TrM1, TrM4, TrM7, TrM6, TC5, TC7, TC2, TrM2, TT3, TT5, Tr3 and Tr9 isolates were resistant to tested antibiotics (Table 4). Heavy metal sensitivity behaviour of isolates presented in

Table 5. Effect of fungicide or herbicide concentrations on the growth of isolates (10^5 cell ml^{-1}) in yeast extract broth.

Isolate	Control (no pesticides)	Fungicide concentration (g 100 seed^{-1})			Herbicide concentration (ml L^{-1})		
		75	150	300	50	100	150
TrM1	124	118.5	83.5	61	84	6.1	1.4
TrM4	175.5	48	39	1	26	11.5	3
TrM7	243.5	41	16	13.5	59.5	25.5	13.5
TrM6	243.5	74	33	9	23	1.2	0.6
TC5	58.5	23.5	11	6	23.5	1.5	1.15
TC7	213	125.5	91.5	81.5	50	0.34	0.011
TC2	123.5	29.5	14	11	80.5	57.5	33
TrM2	212	74	35	1.5	35	1.05	0.3
TT3	244.5	119	83.5	42	30.5	18.5	0
TT5	311	107.5	83.5	71.5	161.5	12.1	0
Tr3	269	26	11	8.5	60	51	0
TrM5	95	19	11	3.5	46	3.25	1.25
Tr9	114.5	43.5	39	26	36	21	7.5
TT4	80	36	33.5	13	68.5	3.95	0.19

ANOVA

	df	Mean square	Mean square
Replication	1	1.08	0.24
Treatment (T)	2	205314.5**	270793**
Control and others	1	389845.3**	499113.7**
Other	2	10391.8**	21236.2**
Isolate (I)	13	10368.5**	5301.2**
I x T	39	2845.6**	3607.13 **
Error	54	4.16**	4.02

**Significant at the 1% probability level.

Table 4 revealed that all the isolates were resistant to one or more heavy metals. The four isolates (TrM1, TrM4, TrM7 and TC5) were resistant to $CdCl_2$ while two isolates (TC7 and TC2) were resistant to $HgCl_2$ (Table 4). Resistance to $CdCl_2$ was also reported by Sheng and Xia (2006), for rhizobial isolates such as *Pseudomonas, Azomonas, Bacillus* and *Arthrobacter* from China soils.

In this study, all the isolates were resistant to $ZnSO_4$. The resistance to heavy metals of rhizobial isolates showed that all the tested isolates (100 %) exhibited resistance to $ZnSO_4$, 28.6% to $CdCl_2$, 21.4% to $CuCl_2$ and 14.2% $HgCl_2$. In the presence of $HgCl_2$, the isolates were sensitive (85.7%) and resistant (14.2%) (Table 4). Similar observations were recorded by Lakzian et al. (2002) and Koppittke et al. (2007). The tested isolates can be grouped into at least six groups; group these isolates, for example;

I) TrM1, TrM4, TrM7 and TC5 resistant to $ZnSO_4$ and $CdCl_2$, sensitive to $CuCl_2$ and $HgCl_2$;
II) TrM6 and TrM2 similar to I, but sensitive to $CdCl_2$;
III) TC7; intermediate to $CuCl_2$, resistant to $ZnSO_4$ and $CdCl_2$; sensitive to $HgCl_2$;
IV) TC2; similar to II, but resistant to $HgCl_2$;
V) TT3, TT5 and Tr3; intermediate to $CuCl_2$, resistant to $ZnSO_4$, sensitive to $CdCl_2$ and $HgCl_2$;
VI) TrM5, Tr9 and TT4 similar to II, but resistant to $CuCl_2$ (Table 4).

In genetic studies, heavy metal resistance traits should be extremely valuable as positive selection markers (Lakzian et al., 2002). Kinraide and Sweeney (2003) suggest that the high levels of Cd, Ar, Al, Cu and La could be used as a selective agent for some *Rhizobium* isolates. Characteristics relating to origin are probably associated with adaptations to specific environmental pressures (Ausili et al., 2002; Giller et al., 1993; Neumann et al., 1998). Pesticides used in agriculture often display toxic effects on various organisms including rhizobia because many metabolic processes are common to all cellular organisms (Allievi and Gigliotti, 2001). The toxic effect of herbicides such as brominal and pyramin to nitrogen fixing bacteria has been reported (Omar and Abd-Alla, 1992).

In the present study, all the isolates were tested for their tolerance to three levels of fungicite or herbicide. The effect of the pesticides and doses on the isolates as variable, depending on the fungicide or herbicide concentrations and isolates are as stated in Table 5. The

highly concentration of fungicide was 99.4 % and 99.2 % inhibited the isolate TrM4 and TrM2, respectively (Table 5). Dose of high herbicide was 100% inhibited by the TT3, TT5 and Tr3 isolates (Table 5). The results of the study were statistically analysed as given in Table 5. The isolates, fungicide concentrations, herbicide concentrations and isolate x treatment interaction mean squares were significant at 1% level. The effect of the fungicide or herbicide concentrations on the isolates was variable, depending on the concentrations and isolate (Table 5).

Increasing concentrations of fungicide or herbicide decreased growth of isolates (Table 5). Growth on fungicide or herbicide concentrations was different from that of the control (without treatment). Herbicide concentrations resulted in a significant decrease in the growth of isolates (Table 5). Also, Castro et al. (1997) and Madhavi et al. (1993) reported that increasing pesticide concentration decreased growth of rhizobial isolates in a dose dependent manner. Madhavi et al. (1993) and Martesson and Nilsson (1989) reported herbicides do not affect rhizobial cell division and the outer wall of the bacterial isolates may have been impermeable to herbicides.

Conclusion

The stress tolerance study of isolates are of potential value for isolate improvement in agriculture. The persistence of pesticides in the soil depends on several factors including soil moisture, temperature, pH, organic matter content, clay content and chemical structure of the pesticides (Kale et al., 1989; Madhavi et al., 1993). However, there is a need to test rhizobial isolates against various herbicides and fungicides commonly used for the control of weeds and pathogenic fungi in all major legumes, for their possible adverse effect on *Rhizobium*.

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