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In vitro Survival and Nematicidal Activity of *Rhizobium, Bradyrhizobium* and *Sinorhizobium.* I. The Influence of Various NaCl Concentrations

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Abstract: During the survey of the cultivated fields in Karachi and neighborhood (Southern Sindh), 3 strains of *Rhizobium phaseoli*, 1 strain of *R. leguminosarum* and *R. trifolii* each, 5 strains of *Sinorhizobium meliloti*, 2 strains of *Bradyrhizobium japonicum* and 3 strains of *Bradyrhizobium* sp. were isolated and identified. The 15 strains of rhizobia tested for their growth under saline media exhibited varying degree of effects to salt concentrations. Most resistant strain was that of *S. meliloti* MAT1(R9) while least resistant was that of *Bradyrhizobium* sp. VRM1(R13). All the rhizobial strains caused significant mortality of *Meloidogyne incognita*, the root-knot nematode juveniles *in vitro,* though the strains differed markedly in their toxic activity. The rhizobial strains showed significant interaction with NaCl salinity towards *M. incognita*.

Key words: Rhizobia, salinity, *Meloidogyne javanica*, variation, juvenile mortality

Introduction

Root-knot nematodes (*Meloidogyne* spp.) are worldwide in distribution and are regarded as one of the most important pests causing severe losses to economically important crops. These important crop pests occur in intensive cropping systems and have until recently been controlled by nematicides. With the withdrawal of nematicides from the market, in particular methyl bromide which will be banned to use in Europe from 2005 (and in developing countries by 2015) alternative approaches are being sought. The application of micro-organisms to the soil as biological control agents offers an alternative method to control plant-parasitic nematodes (Siddiqui and Ehteshamul-Haque, 2001). To date the application of fungi and bacteria to control these nematodes has produced highly variable results with successes in one situation but not in other. The inconsistent performance of these microorganisms is attributed to differences in soil physical and chemical properties. Understanding the factors that regulate the biosynthesis of nematicidal compounds by bacteria is an essential step towards improving the level and reliability of their biocontrol activity.

Rhizobia are soil bacteria which display symbiotic interactions with specific legume hosts. Most of these bacteria are very sensitive to soil water deficit, which adversely affects their nitrogen fixation capacity and hence the productivity of the legume plant (Miller, 1996). It has been estimated that 23% of agricultural soils are affected by problems related to high salinity. Most crops are sensitive to relatively low levels of salinity, and, in the case of legumes, there is an additional problem because not only the plants but also the symbiotic bacteria are sensitive to salinity both at the free living stage and during the symbiotic process (Lloret, 1995). Application of Rhizobia in highly saline soils is considered ideal because some strains can withstand relatively high osmotic conditions (Miller, 1996).

Liquid culture screening is an attractive alternative approach for identifying putative environmental signals because it requires little knowledge of biosynthetic loci and because it is more adaptable to the simultaneous detection of multiple metabolites. This is an important advantage because many of the most effective biocontrol strains produce several antimicrobial compounds, the relative importance of which probably depends on the types of soil, host and pathogen; the stage of disease development; and other environmental conditions (Thomashow and Weller, 1996; Voisard *et al.*, 1994). Recent studies suggest that factors identified *in vitro* by using liquid culture screening do indeed act as important environmental signals in natural habitats. The aim of the present study was to investigate the effect of various concentrations of salts on growth and subsequent nematicidal activity of rhizobia *in vitro*.

Materials and Methods

During a survey of the cultivated fields of Southern Sindh in February-May 2000, 15 strains of Rhizobia belonging to 4 species were isolated from different leguminous plants. Strains of *Rhizobium*, *Bradyrhizobium* and *Sinorhizobium* species isolated in the present study are listed in Table 1. The bacteria were maintained on an enriched semi-selective medium. The enriched medium consisted of 5g of mannitol sugar, 0.25g of K_2HPO_4, 0.1g of $MgSO_4 \cdot 7H_2O$, 2g of $CaCO_3$, 0.05g of NaCl, 0.2g of yeast extract and 7.5g of Agar. These ingredients were dissolved in 500ml distilled water and the pH was maintained between 6.8 and 7.0 before autoclaving. To determine the effects of various concentrations of salt (NaCl) on survival and growth of Rhizobia, a loopfull of each bacterium was transferred in 250ml capacity Erlenmeyer flasks each containing 100 ml enriched liquid medium (broth) with the same nutrients as above but with different salt concentrations respectively: 0, 0.5, 0.25, 0.12, 0.06, 0.03M. The flasks were incubated at room temperature and were allowed to grow for one week at 28°C without shaking. Each bacterial culture was divided into two equal halves. One of the half was tested for the bacterial colony counts while the other half was checked for the nematicidal activity. To determine the bacterial cfu, one ml of the suspension was transferred in a test tube containing 9 ml sterile distilled water and shaken well. A serial dilution was then prepared and 0.5ml from appropriate dilution was plated onto semi-selective agar plates as described above. The plates were incubated at room temperature for 48h and number of cfu recorded.

For the preparation of culture filtrate, the bacterial cells were centrifuged twice (4,500 x *g*, 15min), pellet was discarded and supernatant was collected in a sterilized beaker. The supernatant was passed through two folds of Whatman No. 1 filter paper and the filtrate collected in a sterilized beaker. Egg masses of the root-knot nematode (*Meloidogyne incognita*) obtained from pure culture maintained on tomato (*Lycopersicon esculentum* Mill.) roots were placed in sterilized distilled water for 48h at room temperature for hatching. Hatched juveniles collected in a beaker were used for *in vitro* test. One ml of the culture filtrate was transferred in watch glasses to which 1ml of freshly hatched larval suspension containing 30-35 surface sterilized juveniles was added. Juveniles kept in nutrient rich broth amended with various concentrations of salt without the bacteria or kept in sterile distilled water served as controls. Each treatment was replicated four times and watch glasses were kept at room temperature. After 48h of incubation, the numbers of dead juveniles were counted and percentage mortality was calculated. The nematodes were considered to be dead if they did not move on probing with a fine needle.

Results

During the survey of the cultivated fields in Karachi and its neighborhood (Southern Sindh), 3 strains of *Rhizobium phaseoli*, 1 strain each of *R. leguminosarum* and *R. trifolii*, 5 strains of *Sinorhizobium meliloti*, 2 strains of *Bradyrhizobium japonicum* and 3 strains of *Bradyrhizobium* sp. were isolated and identified (Table 1). Species and even strain specific differences were observed with respect to their growth and survival in the nutrient rich medium. In general with the increasing concentration of NaCl, the growth of all the rhizobial isolates, with the exception of MAT1(R9), declined (Table 2). The effect was accentuated at highest concentration of NaCl (0.5M). *R. phaseoli* strain PVTI(R2) isolated from root nodules of *Phaseolus vulgaris* and *Sinorhizobium meliloti* strain MAT1(R9) from *Melilotus alba* growing in Thatta and *S. meliloti* strain MAG1(R10) isolated from *M. alba* growing in Gharo exhibited high degree of tolerance to salinity and withstood salinity level of 0.5M NaCl concentration. Rhizobial isolates exhibited considerable differences in causing juvenile mortality of *Meloidogyne. incognita* (Table 3). Rhizobial isolates significantly (p at the most 0.05) increased mortality over the controls. Salt concentration had a differential effect on *M. javanica* mortality. In general, juvenile mortality of *M. incognita* increased with increasing NaCl concentration. Salinity and rhizobial strains showed significant interaction ($p<0.01$). Some isolates like PSG1(R4), MIG1(R7), MAT1(R9)and MAG1(R10) showed pronounced increase in mortality with increasing salinity level while others such as PVT1(R2), PLG1(R3), and MIM1(R6) did not show marked difference in juvenile mortality with the rise in salinity.

Table 1: List of the species and strains of *Rhizobium, Bradyrhizobium* and *Sinorhizobium*.

Rhizobium spp.	Strain No.	Source	Locality
Rhizobium phaseoli	PVG1(R1)	*Phaseolus vulgaris*	Gharo
R. phaseoli	PVT1(R2)	*Phaseolus vulgaris*	Thatta
R. phaseoli	PLG1(R3)	*P. lunatus*	Gharo
R. leguminosarum	PSG1(R4)	*Pisum sativum*	Gharo
Rhizobium trifolii	TST1(R5)	*Trifolium* sp.	Thatta
Sinorhizobium meliloti	MIM1(R6)	*Melilotus indica*	Malir
S. meliloti	MIG1(R7)	*M. indica*	Gharo
S. meliloti	MIK1(R8)	*M. indica*	KUcam*
S. meliloti	MAT1(R9)	*M. alba*	Thatta
S. meliloti	MAG1(R10)	*M. alba*	Gharo
Bradyrhizobium japonicum	GMK1(R11)	*Glycine max*	KUcampus
B. japonicum	GMG1(R12)	*Glycine max*	Gharo
Bradyrhizobium sp.	VRM1(R13)	*Vigna radiate*	Malir
Bradyrhizobium sp.	VRK1(R14)	*V. radiate*	KUcampus
Bradyrhizobium sp.	VMG1(R15)	*V. mungo*	Gharo

*Karachi University campus.

Table 2: Effect of various salt concentrations on colony forming units of the species of *Rhizobium, Bradyrhizobium* and *Sinorhizobium in vitro*.

Rhizobial strains	Cfu of rhizobia/ml [$\log_{10}(x+1)$] Salt concentration (M)					
	0	0.030	0.06	0.12	0.25	0.5
PVG1(R1)	9.58	9.89	8.78	8.18	8.37	8.22
PVT1(R2)	9.36	9.78	9.41	9.31	9.21	9.04
PLG1(R3)	8.88	9.05	9.02	8.93	8.15	7.88
PSG1(R4)	9.08	9.15	8.82	8.57	8.48	8.08
TST1(R5)	9.61	9.88	9.09	9.15	8.92	8.43
MIM1(R6)	8.46	8.69	8.93	8.59	8.08	8.21
MIG1(R7)	9.01	9.10	8.82	8.77	7.96	8.28
MIK1(R8)	9.19	9.27	8.31	8.65	8.24	7.88
MAT1(R9)	9.25	9.68	9.07	9.24	9.33	9.21
MAG1(R10)	9.38	9.59	9.41	9.11	9.35	9.19
GMK1(R11)	9.18	9.57	9.12	9.34	9.21	8.38
GMG1(R12)	8.92	9.31	8.59	8.51	8.39	8.04
VRM1(R13)	9.45	9.62	9.29	8.94	8.54	7.92
VRK1(R14)	9.37	9.62	9.20	9.07	8.92	8.48
VMG1(R15)	8.89	9.13	9.00	8.64	8.29	7.65
$LSD_{0.05}$						
Strains			0.81			
Salt conc.			0.58			

Table 3: Effect of various salt concentrations on mortality of *Meloidogyne incognita* juveniles by *Rhizobium, Bradyrhizobium* and *Sinorhizobium in vitro;* Control (1) = sterile distilled water and control(2) = nutrient rich liquid medium

Rhizobial strains	Juvenile mortality (%) Salt concentration (M)					
	0	0.030	0.06	0.12	0.25	0.5
Control(1)	2	4	3	6	10	19
Control(2)	13	15	16	9	13	23
PVG1(R1)	37	18	34	43	33	39
PVT1(R2)	37	44	51	46	38	34
PLG1(R3)	15	11	25	19	22	20
PSG1(R4)	17	49	55	58	41	47
TST1(R5)	30	38	55	68	41	35
MIM1(R6)	21	28	31	50	29	44
MIG1(R7)	27	35	38	41	57	48
MIK1(R8)	26	22	27	17	33	29
MAT1(R9)	23	39	44	34	37	49
MAG1(R10)	28	33	39	35	38	41
GMK1(R11)	29	27	33	44	41	46
GMG1(R12)	34	51	47	51	58	52
VRM1(R13)	25	47	61	38	51	46
VRK1(R14)	20	43	45	51	46	56
VMG1(R15)	18	24	19	32	41	29
$LSD_{0.05}$						
Strains			16			
Salt conc.			13			

Discussion

A change in salt concentration alters the osmotic potential of the rhizosphere and affects the growth and functioning of rhizobacteria. In a previous report, exposure of *P. fluorescens* strain CHA0-Rif to 0.7M NaCl *in vitro* had no effect on subsequent persistence of the cells in soil, whereas incubation in the presence of 1.5M NaCl resulted in non-culturable cells both *in vitro* and subsequently in soil (Mascher *et al.*, 2000). Likewise, the culturability of cells of *P. fluorescens* strain AH9 was reduced after incubation in 1.7M NaCl but not after incubation in 1M NaCl (Jørgensen *et al.*, 1994). In contrast, *P. aeruginosa* PAO1 (Velasco *et al.*, 1995) and *Escherichia coli* (Roth *et al.*, 1988) were affected at much lower concentrations of 0.7 and 0.8M NaCl, respectively. This may, in part, reflect differences in osmotic potential between the habitats from which these bacteria originated. Interestingly, rhizobial species isolated from Karachi and Malir were more sensitive to high salt concentrations compared to those isolated from Thatta or Gharo. Most of the soils of Thatta and Gharo are highly saline and water-logged. Therefore, these strains seem to be well adapted to highly saline conditions. Tolerance to high NaCl concentrations is suggested to be an important bacterial property for successful colonization of the root (Loper *et al.*, 1985; McInnes *et al.*, 1994).

Results obtained here indicate significant differences in the growth pattern of different rhizobial species. Furthermore, the rhizobial strains differed greatly in the nematicidal activity towards *M. incognita*. A halotolerant strain of *Rhizobium* (*Sinorhiobium*) has been isolated from the nodules of *Melilotus alba* growing in a salt marsh in Donana National Park in the southwestern region of Spain. This strain is able to grow at NaCl concentrations of up to 0.5 M (Lloret *et al.*, 1995). The results of the present study also show the capability of *Rhizobium* strains MAT1(R9) to grow at 0.5 M salt concentration, but exhibited better growth at lower salt concentrations. Previous research has shown that changes in osmotic concentrations and pH change the structure of lipopolysaccharides of bacteria in response to salt stress, and that rhizobia accumulate several compatible solutes to overcome the osmotic stress induced by salt. An example of this feature is ectoine, which exhibits osmoprotective properties without being accumulated (Talibart, 1994).

In the present study, cell free culture filtrate of some rhizobial strains caused mortality of *M. javanica* juveniles. Rhizobia which is known to produce rhizobitoxin (Chakraborty and Purkayastha,

1984) has shown promising results in the control of soilborne root-infecting fungi in okra (Siddiqui *et al.*, 2000) and *M. javanica*, the root-knot nematode in mungbean (Siddiqui *et al.*, 1998). Nematicidal activity was greatly affected when the growth medium was amended with high salt concentrations (0.25 and 0.5 M). These results suggest that whereas a low salt concentration enhanced bacterial metabolism, a high salt concentration repressed the synthesis of nematicidal principles.

In the present study, a 0.25 and 0.5M NaCl without bacterial inoculation also caused substantial mortality of *M. incognita* juveniles. Egg hatching of *M. javanica* decreased as the concentration of the electrolytes (NaCl, $CaCl_2$, KCl) increased in the solution media (Dropkin *et al.*, 1958). Likewise, *Heterodera schachtii*, cyst nematode egg hatching (Ellenby and Gilbert, 1958), and reproduction of free living nematodes (Everard, 1960), were decreased when exposed to saline media. Edongali *et al.* (1982) reported that infectivity and development of *M. incognita* on tomato was impaired by increasing soil solution concentrations of NaCl, $CaCl_2$ or combinations of the two salts.

The osmolality of rhizosphere soil water is expected to be elevated in relation to bulk-soil water osmolality as a result of the exclusion of solutes by plant roots during water uptake, the release of plant root exudates, and the production of exopolymers by plant roots and rhizobacteria. In contrast, the osmolality of water within highly hydrated bulk soil is low (less than 50 Osm/kg); thus the ability to adapt to elevated osmolality is likely to be important for successful rhizosphere colonization by rhizobacteria. Result of the present study would therefore suggest that a biocontrol strain of rhizobia with high salt tolerance could be exploited in the practical agriculture for the suppression of plant-parasitic nematode particularly in those areas where the soils are saline and water-logged.

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