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Zinc Improves Biocontrol of *Meloidogyne javanica* by the Antagonistic Rhizobia

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Abstract: Mineral amendments influence the performance of antagonistic microorganism to suppress soil-borne fungal and nematode diseases. Experiments were conducted to evaluate the influence of zinc on the production of nematicidal compound(s) *in vitro* and root-knot infection by *Meloidogyne javanica* in tomato. Nutrient rich medium amended with various concentrations (0.25-2.0 mM) markedly improved the nematicidal activity of rhizobia *in vitro*. Species and even strain-specific differences were observed among bacteria with respect to their response to different zinc concentrations. Efficacy of the 10 different isolates (66.6% of the total isolates) was maximum when growth medium was amended with zinc at 1.5 mM while 4 isolates (26.6% of the total isolates) exhibited optimal performance when exposed to 2.0 mM zinc. *In vitro* nematicidal activity of only one strain was optimal at 1.0 mM zinc. Soil amendment with zinc in the form of $ZnSO_4$ at 0.9 mg/kg of soil alone or in conjunction with rhizobia caused significant inhibition of root-knot development and enhanced the growth of tomato plants under glasshouse conditions.

Keywords: *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, $ZnSO_4$, *Meloidogyne javanica*, biocontrol

Introduction

Micro- and macroelement amendments have been used commercially on a limited scale to manage certain soil-borne diseases (Engelhard, 1989). Disease reduction is most often attributed to improved nutrition that boosts host defenses or to direct inhibition of pathogen growth and activity. Pathogen suppression may also result indirectly from amendment-mediated modification of chemical and physical properties like soil and rhizosphere pH (Simon and Sivasithamparam, 1989) or from modification of host root exudates to disfavour pathogenic activity (Huber, 1989). In a few cases, though, mineral amendments appear to reduce disease by indirectly stimulating indigenous populations of microorganisms that are beneficial to plant growth and antagonistic to pathogens (Huber, 1989).

There is an increasing interest in the introduction of bacterial and fungal biocontrol agents for managing soil-borne pathogens, partly as a response to public concerns about non-target effects of synthetic pesticides and fumigants, but also because of lack of effective controls for soil-borne pathogens (Cook, 1993). However, many biocontrol agents are inconsistent in their performance from site to site and this has been a primary obstacle to their commercial development. Understanding the source of variability is a key factor to overcoming this obstacle. In many cases this variation in the biocontrol performance has been attributed to changes in biotic and abiotic factors associated with field location and cropping time (Duffy and Défago, 1997). Complex abiotic factors including alteration in soil physical and chemical properties by the amendments of specific nutrient, influence the biocontrol of introduced bacteria. A one time amendment of zinc-EDTA at 33 μg of Zn^{2+}/ml to hydroponic nutrient solution in soilless rockwool culture did not reduce crown and root rot disease caused by *Fusarium oxysporum* f. sp. *radicis lycopersici* when use alone, but did reduce disease by 25% in the presence of *Pseudomonas fluorescens* strain CHA0 (Duffy and Défago, 1997). Similarly, in a previous study, micro-elements including zinc in low amounts enhanced the biocontrol potential of plant growth promoting rhizobacterium, *Pseudomonas aeruginosa* against root-knot nematode (*Meloidogyne javanica*) (Siddiqui *et al*., 2002).

Bacteria of the genera *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, *Allorhizobium*, *Mesorhizobium* and *Azorhizobium*, commonly known as rhizobia, are of great environmental and agricultural significance because their symbiosis with legumes is responsible for most of the fixation of atmospheric nitrogen on earth. These microorganisms are soil bacteria able to elicit the formation of new organs, called nodules, on most species of the family *Fabaceae* and on the non-leguminous *Parasponia*, in which they reduce atmospheric nitrogen to ammonia to benefit the host plant (Chaintreuil, 2000). Apart from their nitrogen fixing capabilities, rhizobia also benefit economically important crops by protecting them from various soil-borne plant pathogens including root-knot nematodes (Siddiqui *et al*., 1998; Siddiqui *et al*., 2000). In

view of the above mentioned dual importance of rhizobia (nitrogen fixation and plant disease suppression), experiments were conducted to evaluate the influence of zinc on potential of some strains of rhizobia in growth enhancement and biocontrol of root-knot nematode under laboratory and glasshouse conditions.

Materials and Methods

Microorganisms and culture conditions: The experiments were conducted at Soil Biology and Ecology Laboratory, Department of Botany, University of Karachi. Rhizobial species and strains used in this study are listed in Table 1. The bacteria were maintained on an enriched semi-selective medium. The enriched medium (EM) consisted of 5 g of mannitol sugar, 0.25g of K_2HPO_4, 0.1g of $MgSO_4 7H_2O$, 2g of $CaCO_3$, 0.05g of NaCl, 0.2g of yeast extract, 7.5g of agar. These ingredients were dissolved in 500 ml distilled water and the pH was maintained between 6.8 and 7.0 before autoclaving. A loopful of bacterial culture (previously multiplied on EM for 2 days) was mixed with EM (broth) amended with various concentrations (0, 0.50, 1.0, 1.5 and 2.0 mM) of Zinc in the form of $ZnSO_4$ and incubated at 28°C for 48 h. For the preparation of culture filtrate, the bacterial cells were centrifuged twice (4,500 x g, 15 min), 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 was collected in a sterilized beaker.

Egg masses of the root-knot nematode (*Meloidogyne javanica*) obtained from pure culture maintained on brinjal (*Solanum melongena* L.) roots were placed in sterilized distilled water for 48 h at room temperature for hatching. Hatched juveniles collected in a beaker and used for *in vitro* and glasshouse experiments.

***In vitro* experiments:** One ml of the culture filtrate was transferred in watch glasses to which 1 ml of freshly hatched larval suspension containing 30-35 surface sterilized juveniles was added. Juveniles were kept in nutrient rich broth amended with various concentrations of zinc without the bacteria or kept in sterile distilled water that served as controls. Each treatment was replicated four times and watch glasses were kept at room temperature. After 48 h of incubation, the number 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.

Glasshouse experiments: Under laboratory conditions, strains *R. leguminosarum* PSG1(R4) and *S. meliloti* MIG1(R7) caused greater mortality of *M. javanica* juveniles *in vitro* therefore, these strains were selected for glasshouse experiments. The culture medium and conditions for the growth of rhizobia were the same as mentioned above. Sandy loam soil (pH 8.1, moisture holding capacity of 40%) was mixed with 0.9 mg Zn kg^{-1} of soil and filled in 8 cm diam plastic pots at 350 g pot^{-1}. After amendment, a 35 ml aqueous cell suspension of PSG1 (R4) or MIG1 (R7) containing 10^8 cfu ml^{-1} was drenched in each pot. A set of bacteria-treated plants without Zn was also kept for comparison. Soil drenched with 35 ml sterile distilled water served as a control. After treatments, 3-week-old tomato (*Lycopersicon esculentum* Mill.) cv. SUN (6002) PVP seedlings that had been raised in sterilized soil were planted at three seedlings pot^{-1}. One week after seedling transplantation, roots were infested with 2000 freshly hatched juveniles of *M. javanica*. Treatments and controls were replicated four times and randomized on a glasshouse bench. The experiment was terminated 44

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

S. No.	*Rhizobium* spp.	Strain No.	Source	Locality
1.	*Rhizobium phaseoli*	PVG1(R1)	*Phaseolus vulgaris*	Gharo
2.	*R. phaseoli*	PVT1(R2)	*Phaseolus vulgaris*	Thatta
3.	*R. phaseoli*	PLG1(R3)	*P. lunatus*	Gharo
4.	*R. leguminosarum*	PSG1(R4)	*Pisum sativum*	Gharo
5.	*Rhizobium trifolii*	TST1(R5)	*Trifolium* sp.	Thatta
6.	*Sinorhizobium meliloti*	MIM1(R6)	*Melilotus indica*	Malir
7.	*S. meliloti*	MIG1(R7)	*M. indica*	Gharo
8.	*S. meliloti*	MIK1(R8)	*M. indica*	KU campus*
9.	*S. meliloti*	MAT1(R9)	*M. alba*	Thatta
10.	*S. meliloti*	MAG1(R10)	*M. alba*	Gharo
11.	*Bradyrhizobium japonicum*	GMK1(R11)	*Glycine max*	KU campus
12.	*B. japonicum*	GMG1(R12)	*Glycine max*	Gharo
13.	*Bradyrhizobium* sp.	VRM1(R13)	*Vigna radiate*	Malir
14.	*Bradyrhizobium* sp.	VRK1(R14)	*V. radiate*	KU campus
15.	*Bradyrhizobium* sp.	VMG1(R15)	*V. mungo*	Gharo

*Karachi University campus.

days after the addition of nematode and plant growth parameters including plant height and fresh weight of shoot were recorded. The galls induced by *M. javanica* were subsequently counted under a low power microscope (x 6).

Statistical analysis: Data were analyzed and subjected to analysis of variance (ANOVA) or factorial analysis of variance (FANOVA) depending upon the experimental design using STATISTICA ver. 5.0 software. The follow up of ANOVA included least significance difference (LSD) test.

Results

Culture filtrates of different rhizobial species obtained from nutrient rich medium amended with various concentrations of zinc caused significant ($p<0.05$) mortality of *M. javanica* juveniles *in vitro* (Table 2). Zinc concentrations had variable influence on the nematicidal activity of different rhizobial species. In general, with the increasing concentration of zinc to the growth medium, nematicidal activity of the rhizobial species increased. *R. leguminosarum* PSG1(R4) caused highest mortality of *M. javanica* juveniles followed by *S. meliloti* MAT1(R9). A Zn concentration of 1.5 mM was optimal for the improved nematicidal activity of ten rhizobial strains (66.6% of the total isolates) including PVT1(R2), PSG1(R4), TST1(R5), MIM1(R6), MIG1(R7), MAT1(R9), MAG1(R10), GMG1(R12), VRM1(R13) and VRK1(R14). A Zn concentration of 2 mM was maximal for the enhancement of nematicidal activity of four bacterial strains (26.6% of the total isolates) including PVG1(R1), PLG1(R3), GMK1(R11) and VMG1(R15). A Zn concentration of 1 mM was optimal for the nematicidal activity of the strain MIK1(R8) (6.6% of the total isolates) (Table 2).

Table 2: Effect of various Zinc 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

	Juvenile mortality (%) Zinc concentration (mM)				
Rhizobial Strain	0	0.5	1.0	1.5	2.0
Control(1)	3	2	2	5	4
Control(2)	9	10	9	12	15
PVG1(R1)	27	24	28	38	33
PVT1(R2)	31	38	45	50	52
PLG1(R3)	18	15	22	32	27
PSG1(R4)	39	52	58	67	74
TST1(R5)	24	47	36	45	48
MIM1(R6)	18	21	27	25	31
MIG1(R7)	33	39	34	51	64
MIK1(R8)	22	25	25	19	21
MAT1(R9)	37	45	44	58	69
MAG1(R10)	21	28	35	33	51
GMK1(R11)	29	27	33	44	41
GMG1(R12)	41	45	42	37	44
VRM1(R13)	21	24	44	29	37
VRK1(R14)	29	35	25	39	42
VMG1(R15)	25	31	29	36	31
$LSD_{0.05}$					
Strains	12				
Zinc conc.	8				

Table 3: Effect of zinc on the efficacy of *R. leguminosarum* PSG1(R4) and *S. meliloti* MIG1(R7) on root-knot development due to *Meloidogyne javanica*, growth of tomato.

Treatments	Galls per root system	Plant height (cm)	Shoot weight (g)
Control	94	13.2	1.3
Zinc	80	14.7	1.7
PSG1(R4)	76	15.0	1.8
MIG1(R7)	69	14.4	2.0
Zinc + PSG1(R4)	64	16.6	2.1
Zinc + MIG1(R7)	58	17.4	2.3
$LSD_{0.05}$	12	1.1	0.4

Soil amendment with Zn at 0.9 mg/kg alone or in conjunction with two rhizobial strains significantly ($p<0.05$) reduced root-knot development in tomato (Table 3). The two bacteria without zinc amendment also reduced root-knot disease. However, the biocontrol efficacy of two rhizobial strains markedly improved when applied in the soil amended with zinc. Zinc and *S. meliloti* strain MIG1(R7) applied together caused maximum (38% reduction compared with the control; $p<0.05$) of root-knot development. Zinc applied alone or in combination with rhizobia significantly ($p<0.05$) increased plant height and fresh weight of shoot. Zinc and *S. meliloti* strain MIG1(R7) used in combination gave maximum plant height (32% increase over controls) and fresh shoot weight (77% increase over controls).

Discussion

In present study rhizobial culture medium amended with various concentrations of zinc enhanced the nematicidal activity of the bacteria *in vitro*. Soil amendment with zinc also reduced root-knot development in tomato roots and as a consequence enhanced plant growth. It is quite clear from this and our previous study (Shaukat *et al*., 2002) that rhizobia produce potential nematicidal compound(s) *in vitro* and that zinc amendments (with appropriate concentrations) in the growth medium enhances the biosynthesis of nematicidal compounds. Increasing antibiotic concentrations with zinc and other amendments may provide a bridge of protection against root-knot nematode with a rapid root invasion that outpace the ability of introduced bacteria to become established in the rhizosphere and commence *in situ* antibiotic production. Zinc and other minerals have the extra benefit of improving the genetic stability in inoculants (Duffy and Défago, 1995). What role does Zn play in the biosynthesis of nematicidal compounds by species of rhizobia remains

to be disclosed? In other bacteria, a number of mineral amendments have been reported to stimulate the production of antimicrobial compounds. Siderophores, particularly salicylic acid, have been implicated in the ability of certain strains to trigger induced resistance in plants (Duffy and Défago, 1995; Maurhofer *et al*., 1998) and increasing their supply via inoculants may be advantageous. Zinc has previously been reported to stimulate the production of pyochelin and pyoverdin in *Pseudomonas aeruginosa* biocontrol strain 7NSK2 (Höfte, *et al*., 1994) and in the plant-associated *Azotobacter vinelandii* (Huyer and Page, 1989). Interestingly, zinc stimulation relieves bacterial siderophore production from iron repression (Höfte *et al*., 1994), that might allow a greater role for siderophores in microbial interactions under iron-sufficient conditions (Loper and Henkels, 1997). In our previous study, zinc and glycerol alone or in combination enhanced nematicidal activity of the culture filtrate of both *P. aeruginosa* IE-6S^{+} and *P. fluorescens* CHA0 *in vitro* (Siddiqui and Shaukat, 2002).

It is not sure whether zinc-mediated enhancement of the biosynthesis of nematicidal compounds and/or enhancement of host resistance by rhizobia constitute the mechanisms in suppression of root-knot nematode under natural conditions. Mandal and Sinha (1992) suggested that zinc and other minerals reduce Fusarium wilt of tomato by inducing host resistance. Zinc soil content has been found to be positively correlated with the biocontrol activity of introduced *P. fluorescens* 2-79 (Weller and Thomashow, 1994). Soil amendment with zinc at 0.8 or 1.6 mg kg^{-1} of soil alone or in combination with *P. aeruginosa* IE-6S^{+} significantly reduced nematode penetration in tomato roots (Siddiqui *et al*., 2002). Whatever the mechanism involved soil amendment with zinc enhances the biocontrol potential of rhizobia against root-knot nematode in crops of commercial and economic importance such as tomato. Trace mineral amendments are an inexpensive way to improve the biocontrol activity of certain bacterial strains. Formulations that efficiently supply minerals to the target strain may further improve their availability and effect on biocontrol, which means lower doses and reduce, costs (Duffy and Défago, 1997).

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