SUPPRESSION OF *MELOIDOGYNE INCOGNITA* BY *PSEUDOMONAS FLUORESCENS* STRAIN CHA0 AND ITS GENETICALLY-MODIFIED DERIVATIVES: II. THE INFLUENCE OF SODIUM CHLORIDE

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Summary. Culture filtrates of the *Pseudomonas fluorescens* strain CHA0 and its diacetylphloroglucinol (DAPG)-overproducing derivative CHA0/pME3424 inhibited egg hatch and induced mortality of *Meloidogyne incognita* juveniles *in vitro*. Nutrient yeast extract broth medium amended with various concentrations of NaCl influenced antagonistic activity of the two strains of the bacteria; their inhibitory effect was enhanced when the growth medium was supplemented with 0.4 or 0.8 M NaCl but a concentration greater than 0.8 M NaCl slightly reduced bacterial activity against the nematodes. Strain CHA89, a DAPG-negative mutant, and strain CHA77, a hydrogen cyanide-negative mutant of the strain CHA0, failed to induce such inhibitory effects on eggs and juveniles of *M. incognita*. Application of *P. fluorescens* strain CHA0, CHA0/pME3424 or CHA77 to a non-sterilized sandy loam soil reduced *M. incognita* population densities in roots and subsequent root-knot development on tomato whereas strain CHA89 did not. Soil amended with NaCl enhanced the efficacy of strains CHA0 and CHA/pME3424 against root-knot nematode on tomato but not strain CHA77. A 0.8 M NaCl concentration was optimal for the biocontrol effectiveness of the bacteria. Neither wild type strain CHA0 nor its transgenic derivatives influenced tomato root growth in soil with or without NaCl. The bacterial strains did not differ markedly in their rhizosphere colonization pattern.

The study of plant-associated bacteria and their antagonistic potential is important not only for understanding their ecological role and interaction with plants, but also for any biotechnological applications, e.g. biological control of plant pathogens (Krechel *et al.*, 2002). Interest in biological control has recently intensified because of imminent bans on effective chemicals such as methyl bromide, widespread development of chemical resistance in pathogens, and a general need for more sustainable disease control strategies. Recent research has focused on rhizobacteria for the control of plant parasitic nematodes (Becker *et al.*, 1988; Hasky-Günther *et al.*, 1998; Siddiqui *et al.*, 2001), with the objective of proposing strategies for inhibition of egg hatch (Westcott and Kluepfel, 1993), degradation of hatching factor (Oostendorp and Sikora, 1990), or production of metabolites toxic to juvenile nematodes (Meadows *et al.*, 1989). One of the difficulties in developing rhizobacteria as a control measure is that the performance of these agents is highly variable. The inconsistency of the results has been attributed to differences in physical and chemical properties found in natural environments where the biocontrol agents are applied (Thomashow and Weller, 1996). Understanding the environmental factors that influence the activity of pathogens and their antagonists can lead to improvement in the level and reliability of biological control.

Based on increasing knowledge of the molecular mechanisms that underlie metabolite production, the deployment of biocontrol agents improved by genetic engineering seems feasible. Cronin *et al.* (1997) observed that the percentage of mobile juveniles of *Globodera pallida* Stone was reduced threefold following their incubation in the presence of the pseudomonad strain F113, both *in vitro* and in soil. These authors, using a transposon-induced 2,4-diacetylphloroglucinol (DAPG)-negative biosynthetic mutant of F113 and its complemented derivative with restored DAPG synthesis, found that the ability of strain F113 to produce DAPG was responsible for the reduction in juvenile mobility. Likewise, Siddiqui and Shaukat (2003) demonstrated that *Pseudomonas fluorescens* (Trevisan) Migula strain CHA0 and its DAPG-overproducing derivatives caused marked reductions in *Meloidogyne javanica* (Treub) Chitw. population densities in roots and subsequent root-knot development in various crops. A DAPG-negative mutant of the strain CHA0 partly lost its ability to reduce root-knot infection. The aim of the present investigation was to determine the influence of various concentrations of sodium chloride (NaCl) on the efficacy of *P. fluorescens* strain CHA0 and its genetically-modified derivatives towards inhibition of (i) egg hatch and juvenile mobility *in vitro*, and (ii) root-knot development induced by *M. incognita* (Kofoid *et* White) Chitw. on tomato.

MATERIALS AND METHODS

Pseudomonas fluorescens strain CHA0 (Stutz *et al.*, 1986) and its genetically-modified derivatives, including CHA0/pME3424, a 2,4-diacetylphloroglucinol (DAPG) overproducing strain, CHA89, a DAPG-negative strain (Siddiqui and Shaukat, 2003), and CHA77, a hydrogen

cyanide-negative strain (Laville *et al.*, 1998), were used in this study. The bacterial strains were routinely cultivated in shaken (110 rpm) cultures in Nutrient Yeast extract Broth (NYB) at 28 °C for 24 h in the dark. Clumping of the cells was reduced by the addition of 0.05% Triton X-100 to the media. For the preparation of culture filtrates, the bacterial strains were grown at 28 °C for 24 h with shaking (150 rpm) in 250-ml Erlenmeyer flasks containing 100 ml of 1/10-strength NYB amended with NaCl at 0, 0.2, 0.4, 0.8 or 1.6 M. The bacterial culture was centrifuged twice at 2,800 x *g* for 20 min. The supernatant was passed through two layers of Whatman No. 1 filter paper and the filtrate collected in 100 ml Erlenmeyer flasks.

To determine the influence of bacteria on *M. incognita* egg hatch, one ml of the culture filtrate was placed into a glass cavity slide to which one ml of egg suspension containing 68 ± 11 surface-sterilized eggs was added. Eggs placed into a 1/10-strength NYB medium served as controls. After a 96 h exposure to bacterial culture filtrate, the numbers of eggs with intact juveniles were counted and the percentage of hatched eggs calculated. To determine the influence of bacterial strains on *M. incognita* juveniles, one ml of the culture filtrate was transferred to a glass cavity slide to which one ml of a freshly hatched juvenile suspension containing 47 ± 8 surface-sterilized juveniles was added. Juveniles in 1/10 NYB medium served as controls. After 48 h exposure, the numbers of immobile juveniles were counted. The nematodes were considered dead if they did not move on probing with a fine needle (Cayrol *et al.*, 1989). The experiments were conducted twice.

The design of the experiment to test the effects of the bacteria on nematode development on tomato was a 5 x 5 factorial with five replications, each with three tomato seedlings. The factors were five NaCl concentrations (0, 0.2, 0.4, 0.8 or 1.6 M) and five bacterial treatments (none, CHA0, CHA0/pME3424, CHA89 and CHA77). A sandy loam soil (pH 8.1, maximum water retaining capacity of 39%) obtained from an experimental field of the Department of Botany, University of Karachi, was mixed thoroughly with the appropriate quantities of NaCl and placed into 8-cm-diam. plastic pots. After treatment with NaCl, a 35-ml aqueous cell suspension of the bacterial inoculants (10^8 cfu/ml, prepared in sterile distilled water) was poured in the pots. Subsequently, 3-week-old tomato (*Lycopersicon esculentum* Mill.) cv. 'SUN(6002)' PVP seedlings were transplanted into each pot, three seedlings per pot. One week after transplanting, 6 ml of suspension containing 2,000 freshly hatched (< 1-week-old) juveniles of *M. incognita* were introduced into the soil via three holes made in the soil around the seedlings. The experiment was terminated 45 days after the addition of the nematodes, and fresh root weight and numbers of galls induced by *M. incognita* were recorded. The root systems were thoroughly washed with running tap water, cut into small segments and divided into two equal portions. To determine nematode penetration, one of the root portions was cut into smaller segments, wrapped in a muslin cloth and dipped in boiling 0.25% acid fuchsin in lactic acid for 3-5 min. Roots were washed in running tap water to remove the excess stain and macerated in a blender for 45 sec. The macerate was suspended in 100 ml of water and *M. incognita* females and juveniles in five samples of 5 ml each were counted with the aid of low power stereomicroscope (x 10). Rhizosphere bacterial populations of strain CHA0 and its derivatives were recovered by placing root samples with adhering soil in a 100 ml Erlenmeyer flask containing 15 ml of 0.1M $MgSO_4$ solution (pH 6.5) plus 0.02% Tween 20. Ten-fold serial dilutions of the suspension were prepared and 100 ml aliquots from the appropriate dilutions were spread on King's medium B (KMB) agar plates. Bacterial colonies were counted after incubation at 28 °C for 48 hrs.

Data were subjected to factorial analysis of variance (FANOVA) using STATISTICA ver. 5.0 software (Statsoft Inc. Tulsa, Oklahoma, USA). Bartlett's tests for homogeneity of variances were performed in the case of repeated experiments to check if the pooling of the data was warranted. Treatment means were separated using Fisher's least significant difference (LSD) tests. Bacterial population counts were transformed to $\log_{10}$ (x+1) prior to analysis to achieve homogeneity of variance.

RESULTS AND DISCUSSION

Culture filtrates of *P. fluorescens* strain CHA0 and of DAPG-overproducing strain CHA0/pME3424 resulting from 1/10-strength NYB medium significantly ($P < 0.05$) inhibited egg hatch of *M. incognita in vitro* compared to the controls (Table I). The inhibitory effect of the strains tended to be more pronounced when the NYB medium was supplemented with NaCl. Strain CHA0/pME3424 tended to cause greater inhibition of egg hatch than strain CHA0. DAPG-negative strain CHA89 and HCN-negative mutant CHA77 failed to inhibit egg hatch with or without the addition of NaCl to the NYB medium.

Culture filtrates of strains CHA0 and CHA0/pME34 24 caused substantial ($P < 0.05$) mortality of *M. incognita in vitro* (Table II). Nematicidal activity of these strains tended to increase when the growth medium was amended with 0.4 or 0.8 M NaCl but concentration beyound 0.8 M NaCl did not increase percent mortality. DAPG and HCN-negative mutants caused mortality levels similar to those found in the controls. NYB medium amended with 1.6 M NaCl without the bacterial culture filtrates also caused significantly ($P < 0.05$) more mortality than the control receiving no NaCl. The results of the *in vitro* tests suggest that biosynthesis of secondary metabolites, including DAPG and HCN, by fluorescent pseudomonads may play an important role in the inhibition of egg hatch and juvenile mortality. Addition of NaCl to the growth medium influenced bacterial metabolism some-

Table I. Influence of culture filtrate of *Pseudomonas fluorescens* strains CHA0, CHA0/pME3424, CHA89 and CHA77 resulting from 1/10-strength Nutrient Yeast extract Broth supplemented with various concentrations of NaCl on egg hatch of *Meloidogyne incognita in vitro*. (Activity was measured 96 h after exposure to bacterial culture filtrate. Values are means of two individual experiments, four replications per treatment).

NaCl concentration (M)	Bacterial strain				
	None	CHA0	CHA0/pME3424	CHA89	CHA77
0	88	58	51	79	75
0.2	92	44	47	85	78
0.4	79	50	38	77	72
0.8	82	42	35	84	80
1.6	75	61	46	79	76
$LSD_{0.05}$					
NaCl concentration (C)			14		
Bacterial application (B)			14		
C x B			23		

Table II. Influence of culture filtrate of *P. fluorescens* strain CHA0, CHA0/pME3424, CHA89 and CHA77 resulting from 1/10-strength Nutrient Yeast extract Broth supplemented with various concentrations of NaCl on *M. incognita* juvenile mortality *in vitro*. (Activity was measured 24 h after exposure to bacterial culture filtrate. Values are means of two individual experiments, four replications per treatment).

NaCl concentration (M)	Bacterial strain				
	None	CHA0	CHA0/pME3424	CHA89	CHA77
0	13	47	56	25	19
0.2	15	58	62	21	22
0.4	17	58	68	24	20
0.8	19	61	74	19	25
1.6	26	49	61	21	23
$LSD_{0.05}$					
NaCl concentration (C)			10		
Bacterial application (B)			10		
C x B			18		

how; the possible role of NaCl on bacterial physiology and growth is yet to be determined.

Soil amended with NaCl alone did not reduce final population densities of *M. incognita* juveniles in roots or degree of root galling on tomato but significantly reduced nematode densities when used in combination with *P. fluorescens* strains CHA0, CHA0/pME3424 or CHA77 (Table III). When the efficacy of the biological control agents was compared, DAPG-overproducing strain CHA0/pME3424 generally tended to produce a greater suppression of the root-knot nematodes. With an increase in NaCl concentration in soil, the efficacy of strains CHA0 and CHA0/pME3424 against *M. incognita* also increased. When compared with the controls, strain CHA89 did not influence nematode survival and infectivity, in either NaCl-amended or non-amended soils. These results suggest that the HCN-negative mutant may reduce *M. incognita* population densities and root-knot infection indirectly, via enhancement of defence mechanisms leading to systemic resistance in plants instead of via a direct effect on *M. incognita* eggs and juveniles. However, in addition to induction of systemic resistance, a possible direct role of DAPG and HCN on *M. incognita* cannot be ruled out since strains CHA0 and CHA0/pME3424 caused nematode inhibition to a greater degree. Further studies are required to prove the mechanism of action of *P. fluorescens*. NaCl-mediated enhancement of biological control performance of *P. fluorescens* strain CHA0 is another important point which needs to be addressed. Since Karachi is located in a semi-arid region where most soils are waterlogged and saline, application of CHA0 for the suppression of root-knot nematode may provide a selective advantage to these bacteria in such soils.

Pseudomonas fluorescens strains had no significant effect on root growth of tomato seedlings (data not presented). A change in soil chemical status following soil amendment with NaCl did not change bacterial activity in this respect. The bacteria strains did not differ significantly in their colonization pattern in tomato (Table IV).

Table III. Influence of NaCl on the biocontrol efficacy of *P. fluorescens* strain CHA0 and its genetically modified derivatives CHA0/pME3424, CHA89 and CHA77 towards *M. incognita* on tomato in pot tests.

Strain	Number of galls/g roots					Number of juveniles/g roots				
	NaCl concentration (M)									
	0	0.2	0.4	0.8	1.6	0	0.2	0.4	0.8	1.6
None	119	110	121	106	93	192	179	185	178	167
CHA0	84	72	65	58	77	157	142	128	113	119
CHA0/pME3424	71	74	61	55	67	131	155	121	84	90
CHA89	102	93	112	97	86	182	167	156	162	162
CHA77	96	88	91	85	81	161	149	158	152	134
$LSD_{0.05}$										
NaCl			18					27		
Bacteria			18					27		

Table IV. Influence of NaCl on rhizosphere colonization [$(\log_{10}(x+1)$] by *P. fluorescens* strain CHA0 and its genetically modified derivatives CHA0/pME3424, CHA89 and CHA77.

Strain	NaCl concentration (M)				
	0	0.2	0.4	0.8	1.6
None	-	-	-	-	-
CHA0	5.64	5.72	5.77	5.42	5.20
CHA0/pME3424	5.33	5.55	5.68	5.33	5.01
CHA89	nd	nd	nd	nd	nd
CHA77	5.59	5.48	5.61	5.06	5.11
$LSD_{0.05}$					
NaCl			0.51		
Bacteria			0.51		

nd, not determined

In general, rhizosphere populations of the bacterial inoculants were slightly less in soil amended with a 1.6 M NaCl concentration. Bacteria populations reached a mean density of log 5 cfu per g fresh root weight, a density that seems sufficient to suppress root-knot nematode.

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