SUPPRESSION OF *MELOIDOGYNE INCOGNITA* BY *PSEUDOMONAS FLUORESCENCE* STRAIN CHA0 AND ITS GENETICALLY MODIFIED DERIVATIVES: I. THE INFLUENCE OF OXYGEN

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Summary. Culture filtrates of the biocontrol strain *Pseudomonas fluorescens* CHA0 and its genetically modified, antibiotic overproducing strain CHA0/pME3424 and antibiotic-deficient strain CHA89 caused substantial mortality of *Meloidogyne incognita* juveniles *in vitro*. The bacterial inoculants incubated in limited oxygen conditions caused greater juvenile mortality compared to the culture filtrate obtained from medium kept at ambient oxygen conditions. Strain CHA0/pME3424 caused greater juvenile mortality compared to CHA0 or CHA89 regardless of the incubation conditions. Under glasshouse conditions, the bacterial inoculants used as a soil drench caused marked reduction in nematode population densities in tomato roots compared to the controls. The three bacteria applied separately in the soil watered from the top caused greater reduction in nematode populations compared to the pots watered from the bottom. Regardless of watering, the greatest reduction in nematode populations was achieved after soil treatment with strain CHA0-pME3424. The bacterial antagonists applied in the soil watered from the top enhanced plant growth and protein contents of the leaf. The three bacterial inoculants did not differ significantly in their colonization pattern regardless of watering method. However, the bacterial populations were significantly ($p<0.05$) higher in the pots watered from the top.

Since the rhizosphere provides the first line of defence for roots against nematode attack, it is generally considered that rhizosphere bacteria are ideal biocontrol agents (Hasky-Günther *et al.*, 1998). Fluorescent pseudomonads belong to the genus recognized as ubiquitous soil microorganism capable of effectively colonizing the roots of different plants. Certain strains of fluorescent pseudomonads are able to suppress a variety of plant diseases caused by soil-borne pathogens, and hence are of considerable agricultural value (Kloepper, 1993). Many species of *Pseudomonas* promote plant growth and reduce the population of deleterious microorganisms (Siddiqui and Ehteshamul-Haque, 2001). During the 1980s work on the mode of action of rhizosphere bacteria with biological control activity began to suggest that some bacterial strains may activate host defence systems based on lack of direct antibiosis of the strains toward pathogens or on correlation of biocontrol with plant growth promotion (Scheffer, 1983). In general, pathogen or chemical induced resistance was associated with local and systemic accumulation of pathogenesis-related proteins in plant tissue (van Loon, 1997). However, Reitz *et al.* (2001) demonstrated that the resistance reaction triggered by a rhizobacterium, *Rhizobium etli* G12, against *Globodera pallida*, was not accompanied by enhanced accumulation of pathogenesis-related proteins such as chitinase and b-1,3-glucanase.

Pseudomonas fluorescens strain CHA0 effectively protected wheat from *Gaeumannomyces graminis* var. *tritici* in glasshouse and field experiments and is active against several other root diseases (Défago *et al.*, 1990). The strain produces several secondary metabolites including hydrogen cyanide, 2,4-diacetylphloroglucinol and pyoluteorin. There is increasing interest in commercial application of genetically modified microorganisms with improved biocontrol properties towards soil-borne plant pathogens. A derivative of *P. fluorescens* CHA0 carrying chromosomal CHA0-DNA cloned in cosmid pME3424 was shown to overproduce the antibiotics 2,4-diacetylphloroglucinol and pyoluteorin and also displays enhanced biocontrol capability in host pathogen system (Schnider *et al.*, 1995).

Environmental factors influence the production of antimicrobial compounds including 2,4-diacetylphloroglucinol in fluorescent pseudomonads. Variation in the biocontrol performance of these bacteria has been attributed to changes of biotic and abiotic factors associated with field location and cropping time (Thomashow and Weller, 1996; Duffy and Défago, 1997). Complex biotic factors, such as plant species, plant age, host cultivar, and infection with the plant pathogen *Pythium ultimum*, can significantly alter 2,4-diacetylphloroglucinol production (Notz *et al.*, 2001). Castric (1983) found that hydrogen cyanide production by *P. aeruginosa* growing in a synthetic medium requires aerobiosis but operated efficiently at low dissolved oxygen concentration. The aim of the present investigation was to determine whether oxygen availability influences the production of nematicidal compounds by *P. fluorescens* strain CHA0 and its genetically modified derivatives CHA0/pME3424 and CHA89 *in vitro* and biocontrol of *M. incognita* in tomato under glasshouse conditions.

MATERIALS AND METHODS

P. fluorescens (Trevisan) Migula strain CHA0 and its genetically modified derivatives CHA0/pME3424 and CHA89 were cultivated in King's medium B (KMB); clumping of the cells was reduced by the addition of 0.05% Triton X-100. For aerobic growth, the bacterium was cultured for four days in 500 ml Erlenmeyer flasks containing 100 ml KMB with vigorous shaking. Severely oxygen-limited (anaerobic) cultures of the bacterium were grown in rubber-stoppered 125 ml bottles with gentle shaking. The oxygen initially present in the medium was consumed by the strictly aerobic cells (Højberg *et al.,* 1999). For the preparation of culture filtrates, the bacterial cells were centrifuged twice (4,500 x *g*, 15 min), the pellet was discarded and the supernatant collected in a sterilized beaker. The supernatant was passed through two layers of Whatman No. 1 filter paper and the filtrate collected in a beaker. One ml of the culture filtrate was spread over KMB plates and incubated for 48 h to determine the presence or absence of the bacterial cells. No bacterial cells were observed in the culture filtrate.

The experiment was a 2 x 4 factorial with six replications. The factors included two oxygen conditions and four bacterial strains including a control. Egg masses of the root-knot nematode, *Meloidogyne incognita* (Kofoid *et* White) Chitw. obtained from a pure culture maintained on tomato (*Lycopersicon esculentum* Mill.) roots were placed in sterilized distilled water for 48 h at room temperature for hatching. Hatched juveniles were collected in a beaker, surface sterilized with 0.5% $Ca(OCl)_2$ for 1 min and used for the *in vitro* test. One ml of the culture filtrate was transferred in watch glasses to which 1 ml of freshly hatched juvenile suspension containing 30-35 surface sterilized juveniles were added. Juveniles kept in one ml KB liquid medium without the bacterium served as controls. The watch glasses were kept at room temperature (28±2 °C). After 48 h of incubation, the numbers of dead juveniles were counted and percentage mortality calculated. The nematodes were considered dead if they did not move on probing with a fine needle.

The soil obtained from the experimental field of the Department of Botany, University of Karachi (sandy loam, pH 8.1, maximum water retaining capacity of 38%) was filled in 8 cm diam plastic pots. The base of each pot was removed and replaced with a coarse nylon mesh. The upper 2 cm soil surface in each pot was removed and an aqueous cell suspension of CHA0 (3.8 x 10^8 cfu ml^{-1}); CHA0/pME3424 (2.6 x 10^8 cfu ml^{-1}) or CHA89 (3.1 x 10^8 cfu ml^{-1}) prepared in 35 ml sterile distilled water was introduced into the soil. Soil drenched with 35 ml distilled water without the bacterium served as a control. After treatment, the surface soil was returned and three-week-old tomato seedlings were transplanted in each pot, three seedlings per pot. One week after transplanting, the roots in each pot were inoculated with 2000 freshly hatched (< 1-week-old) juveniles of *M. incognita*. The pots were watered from the bottom on the third day and then every three days by sinking the bottom in a deep container filled with water until the water levels in the pots reached the soil surface. The pots were then lifted, and excess water drained away through the nylon mesh. The pots were watered from the top every three days; the pots were watered until water began to drain through the bottom of the pots. Watering from the bottom causes a depletion of oxygen supply in the pots while those watered from the top were well aerated. There were five replicates for each treatment and pots were randomised.

The experiment was terminated 45 days after the addition of nematodes and plant growth parameters including plant height and fresh weight of shoots and roots were recorded. The root systems growing in a pot after thorough washing with running tap water were cut into small segments and divided into two equal portions. To determine nematode penetration, one of the portions of the fresh roots was cut into small segments, re-weighed in a muslin cloth and dipped for 3-5 min in boiling 0.25% acid fuchsin with lactic acid. Roots were washed in running tap water to remove the excess stain and macerated in an electric grinder for 45 sec. The homogenate was suspended in 100 ml water and *M. incognita* females and juveniles in five samples of 5 ml were counted with the aid of a low power stereomicroscope (x 6). To determine the protein contents, fresh leaves were ground with a pestle and mortar and then extracted with 1 ml of 0.05 M K-Na phosphate pH 7.2, 40 µg/ml phenylsulfonyl fluoride, and 0.01% SDS. The protein content of the supernatant was estimated by the method of Bradford (1976). Rhizosphere populations of the 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 50 µl aliquots from the appropriate dilutions were plated on to KMB agar plates supplemented with appropriate quantities of streptomycin.

Data sets were subjected to factorial analysis of variance (FANOVA) using STATISTICA ver. 5.0 software. The follow up of FANOVA consisted of least significant difference (LSD) test. Bacterial population counts were transformed to $\log_{10}(x+1)$ prior to analysis.

RESULTS AND DISCUSSION

The culture filtrates of *P. fluorescens* strain CHA0 and its genetically modified strains CHA0/pME3424 or CHA89 caused significant ($p<0.05$) mortality of *M. incognita* juveniles *in vitro* (Table I). Regardless of the culture conditions (low oxygen or ambient oxygen), strain CHA0/pME3424 caused greater juvenile mortality compared to CHA0 or CHA89. However, the bacterial inoculants kept at limited oxygen conditions resulted

Table I. Effect of culture filtrates of *Pseudomonas fluorescens* strain CHA0 and its genetically modified strain CHA0-pME3424 and CHA89 incubated at limited oxygen conditions or ambient oxygen conditions on mortality of *Meloidogyne incognita* juveniles *in vitro*.

Bacterial strains	Mortality (%)
Limited oxygen	
Control	8
CHA0	62
CHA0-pME3424	71
CHA89	36
Ambient oxygen	
Control	10
CHA0	46
CHA0-pME3424	57
CHA89	24
$LSD_{0.05}$:	
Bacterial strains (BS)	15
Oxygen availability (OA)	11
BS x OA	26

Means followed by the same letters in each column are not significantly different according to Duncan's multiple range test; $p< 0.05$.

in greater juvenile mortality compared to ambient oxygen conditions. These results clearly suggest that strain CHA0 and its genetically modified derivative produce unknown nematicidal compound(s) in the culture media, secretion of which is largely influenced by oxygen availability. An antibiotic over-producing strain CHA0/pME3424 causes juvenile mortality to a greater extent compared to the wild type strain CHA0 or antibiotic-deficient strain CHA89. Since strain CHA0 is a strictly aerobic organism that needs sufficient oxygen in the media for growth where it produces only a limited amount of nematicidal compounds. Thus, it seems likely that under stressed growth conditions such as low oxygen availability, certain bacterial strains synthesize large amounts of toxic metabolites inhibitory to root-knot nematode juveniles. In our previous study, culture filtrate of *Pseudomonas aeruginosa* strain IE-6S$^+$ obtained from limited oxygen growth conditions caused substantial mortality of *M. javanica* juveniles *in vitro* compared with the filtrates obtained from ambient oxygen conditions (Siddiqui and Shaukat unpubl). In that same study, culture filtrate from limiting oxygen conditions, showed the presence of hydrogen cyanide, a sole or primary toxic factor produced by *P. aeruginosa* that is responsible for killing of the nematode, *Caenorhabditis elegans*.

Under glasshouse conditions, the three bacterial inoculants significantly ($p<0.05$) reduced nematode populations in roots in pots watered from top compared with the untreated controls while only strain CHA0/pME34242 caused significant reduction in nematode invasion in the pots watered from the bottom (Table II). Regardless of the watering, strain CHA0/pME3424 caused greater reduction in nematode populations compared to CHA0 or CHA89. Regardless of the bacterial applications, nematode penetration was greater in the pots watered from the top. Bacteria applied in the soil watered from the top enhanced plant height and the fresh weight of shoots compared to the controls while fresh root weights remained unaltered. Regardless of the two water regimes, application of the strain CHA0 resulted in the greatest plant height and fresh weight of shoot compared to CHA0/pME3424 or CHA89. Similarly, the bacterial inoculants applied in the pots watered from the top significantly ($p<0.05$) increased protein contents of the leaf. However, leaves of plants grown in soil treated with strain

Table II. Effect of two watering regimes (top and bottom) on the efficacy of *P. fluorescens* strain CHA0 and its genetically modified strains CHA0-pME3424 and CHA89 on invasion of *M. incognita*, growth of tomato and bacterial colonization in the rhizosphere.

Treatments	Nematode Population/ g root	Plant height (cm)	Shoot weight (g)	Root weight (g)	Leaf protein mg/g	Bacterial Log_{10} (x+1) rhizosphere
			Top watering			
Control	158	11.8	0.74	0.62	4.57	0
CHA0	126	13.7	0.98	0.58	4.87	5.33
CHA0-pME3424	102	12.8	0.95	0.53	4.92	5.41
CHA89	135	12.3	0.92	0.75	4.78	5.26
			Bottom watering			
Control	130	10.4	0.63	0.57	4.25	0
CHA0	119	11.0	0.68	0.61	4.36	4.29
CHA0-pME3424	108	10.8	0.57	0.48	4.39	4.32
CHA89	125	10.9	0.64	0.56	4.32	4.25
$LSD_{0.05}$:						
Treatments (T)	21	1.8	0.20	0.17	0.19	0.32
Watering (W)	15	1.1	0.11	0.14	0.13	0.27
T x W	33	2.6	0.29	0.26	0.27	0.48

CHA0/pME3424 had greater protein contents of the leaf compared to the plants grown in soil treated with CHA0 or CHA89. Although protein contents of the tomato leaves were markedly higher in bacteria-treated plants, further biochemical characterization of this protein is needed to ascertain whether the elevated levels are due to the production of pathogenesis-related proteins such as chitinase or β-1,3-glucanase. Physiological processes such as enhanced accumulation of pathogenesis-related protein in plant tissues and, increased peroxidase activity and enhanced lignin biosynthesis are known to be commonly expressed by plants in response to nematode invasion (Zacheo and Bleve-Zacheo, 1995). PR proteins trigger resistance reaction in plants against certain soil-borne plant pathogens including plant-parasitic nematodes (Nandi *et al.,* 2002).

No significant differences were observed in the final population densities among the three bacterial strains in the rhizosphere regardless of watering mode. This suggests that enhanced biocontrol activity of the strain CHA0/pME3424 was not related with an increased population of the agents in the rhizosphere. However, for all the strains populations were significantly ($p<0.05$) greater in the rhizosphere watered from the top.

The results of the glasshouse experiment do not correspond with those obtained *in vitro*. This implies that under natural conditions, the bacteria should colonize in the rhizosphere to a final population density of 10^5 cfu or more for the effective control of plant-parasitic nematodes. Furthermore, in addition to the production of nematicidal compound(s), *Pseudomonas fluorescens* also enhances the plant defence mechanisms leading to induction of systemic resistance against root-knot nematode. Although statistically significant, the level of nematode control achieved in the present study by the biocontrol bacteria is not substantially high (36% in the most effective treatment) and thus there are reservations as to its possible exploitation under field conditions. Nonetheless, such *P. fluorescens* strains can be exploited practically for the control of plant-parasitic nematodes through the application of integrated control strategies by combining their application together with various control measures including soil organic amendments, nematophagous fungi and certain beneficial cultural practices.

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