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EXOPOLYSACCHARIDE OVERPRODUCING VARIANT OF *PSEUDOMONAS FLUORESCENS* STRAIN CHA0 ENHANCES TOLERANCE TO VARIOUS ENVIRONMENTAL STRESSES *IN VITRO* BUT DOES NOT IMPROVE *MELOIDOGYNE JAVANICA* BIOCONTROL IN TOMATO

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ABSTRACT

A variety of stress situations may affect the activity and survival of plant-beneficial pseudomonads added to soil to control root diseases. *Pseudomonas fluorescens* strain CHA0 produced extracellular proteases and caused substantial mortality of the juveniles of *Meloidogyne javanica,* the root-knot nematodes, *in vitro* while strain CHA211, a highly mucoid (exopolysaccharide overproducing) derivative of the strain CHA0, did not. In general, the both the bacterial inoculants exhibited osmosensitivity of a similar degree upon exposure to NaCl or sorbitol, strain CHA211 survived better than CHA0 when growing cultures of the bacteria were exposed to sorbitol at 1.2 M concentration. Whereas both the bacterial strains were equally susceptible to paraquat and NaOCl, strain CHA211 was more tolerant to H_2O_2 than CHA0. Likewise, mutant withstood thermal stress (42 and 50°C) better than its wild type counter part CHA0. Application of strains CHA0 or CHA211 to sandy loam soil resulted in a significant reduction of nematode population densities in roots but only strain CHA0 reduced root-knot infection due to *M. javanica*. The bacterial strains did not differ markedly in their colonization in tomato rhizosphere. Strain CHA0 caused systemic reduction of nematode penetration greater than CHA211.

Key words: fluorescent pseudomonads, exopolysaccharide, osmotic/oxidative/ thermal stress, root- knot nematode.

INTRODUCTION

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 many biotechnological applications, e.g., biological control of plant pathogens. Disease suppression by these bacteria involves a variety of mechanisms, including effective competition for nutrients and colonization sites, pathogen inhibition by the production of secondary metabolites, and enhancement in defence mechanism leading to systemic resistance in the plant (Bloemberg and Lugtenberg, 2001; Keel and Défago, 1997; Siddiqui and Shaukat 2002). Following introduction into soil, the biocontrol performance of pseudomonads depends largely on their ability to maintain stable populations and to be metabolically active at least over the period needed to exert their beneficial effects (Weller, 1988; Bull *et al.*, 1991; Johnson, 1994). However, in soil these bacteria are exposed to a range of variable biotic and abiotic stress factors, such as desiccation, competition, predation, and changes in temperature, high osmolarity, and availability of water and nutrients (Van Veen *et al.*, 1997; Miller and Wood, 1996; Schnider-Keel *et al.*, 2001). Therefore, the sizes of introduced pseudomonad populations may decline considerably within a few weeks that often lead to variable biocontrol activity (Thomashow and Weller 1995).

Many root-colonizing pseudomonads, including the phytopathogens, have a mucoid appearance that is typical of abundant production of exopolysaccharide (EPS) alginate, a copolymer of *O*-acetylated -1,4-linked D-mannuronic acid and its C-5 epimer, L-guluronic acid. These polymers may mediate adhesion to surfaces and protection from adverse environmental conditions. Previous studies demonstrated that specific rhizobacteria reduce plant infection by various parasitic nematodes (Oostendorp and Sikora, 1990). Recently, it was shown that the rhizobacterium *Rhizobium etli* strain G12 impaired infection by the potato cyst nematode *Globodera pallida* indirectly by inducing systemic resistance (Hasky-Günther *et al.*, 1998). Since both living and heat-killed cells of the strain G12 enhanced the plant defense capacity in potato roots, these authors concluded that heat-stable surface structures such as exopolysaccharides (EPS) and/or lipopolysaccharides (LPS) act as inducing agents. It is therefore plausible that enhanced EPS production provides bacterial inoculants with a selective advantage for surviving under harsh conditions in soil (Schnider *et al.*, 1997) and causing systemic reduction of nematode infection.

Our principal objective was to determine the influence of certain environmental stresses on the survival of *P. fluorescens* strain CHA0 and its mucoid (EPS overproducing) mutant CHA211. The second objective was to investigate the biocontrol potential of the bacterial strains, which vary in metabolism and cell surface structure (i.e., EPS), towards *Meloidogyne javanica*, the root-knot nematode in tomato. *M. javanica-P. fluorescens*-tomato system was selected here because: (i) tomato is highly susceptible to root-knot nematode; (ii) *P. fluorescens* strain CHA0

has given substantial control of root-knot nematode in tomato (Siddiqui and Shaukat, 2002) and, (iii) due to arid region, most agricultural soils of the southern Sindh, Pakistan are highly saline and due to sandy nature, dry out quickly, while the soil temperature rises to as high as 50°C during the hot summer season. Resistance to such stresses may enhance inoculant's survival in the crop rhizosphere and improve biocontrol effectiveness against soil-borne plant pathogen(s).

MATERIALS AND METHODS

Organisms and culture conditions

Pseudomonas fluorescens strain CHA0 and its mutant strain CHA211 (*mucA*::Tn*5*), which is highly mucoid owing to overproduction of exopolysaccharides (Schnider *et al.* 1997; Schnider-keel *et al.*, 2001), were used in all the experiments. Southern hybridization indicated that mutant CHA211 contained a single Tn*5* insertion (Schnider-Keel *et al.*, 2001). The Tn*5* insertion was located 550 bp downstream of the initiation codon of the *mucA* gene. The mucoid phenotype of the mutant strain, CHA211, was stable, even when the strain was repeatedly subcultured in King's B (KMB; King *et al.* 1954) or nutrient agar media for more than a week. The wild-type strain CHA0 is originally non-mucoid and produces a variety of secondary metabolites. The bacterial strains were routinely grown overnight at 28°C with mild shaking (85 rpm) in 1/10-strength KMB broth. The bacterial cells were centrifuged at 2,800 x *g* for 20 min. The supernatant was passed through sterile Millipore (0.2 μm) filter membrane and cell-free filtrate collected in 100 ml Erlenmeyer flasks. The resulting filtrate was used to assess nematicidal activity (see below). The pellets were resuspended in sterile $MgSO_4$ (0.1 M) to use in the glasshouse experiments. All the laboratory experiments were conducted in August 2002 while glasshouse experiments were conducted in November 2003.

Qualitative determination of protease from bacteria

Assay to determine the production of extracellular protease qualitatively by *P. fluorescens* strains CHA0 and CHA211 was performed as described previously (Pierson *et al.*, 1998). Briefly, enzyme production was measured by spotting 5-μl portions of overnight cultures of test strain on skim milk agar (Difco Laboratories). Plates were incubated at 28±2°C for 24 h. Formation of a zone of clearing around a colony indicated that extracellular protease was produced.

Determination of nematicidal activity

To determine the nematicidal activity one ml of the filtrate was transferred to a cavity glass slide to which one ml of the freshly hatched juvenile suspension containing 53 ± 9 surface-sterilized juveniles were added. Juveniles kept in 1/10-strength KMB broth without the bacterium served as controls. The suspensions were kept at 28°C. Treatments and controls were replicated four times each. After 48 h, the numbers of dead juveniles were counted and mortality percentage calculated. The nematodes were considered dead if they did not move on probing with a fine needle. The experiment was performed twice.

Effect of osmotic stress on bacteria

To induce osmotic stress, KMB broth was supplemented with NaCl (0.6 or 0.8 M) or sorbitol (1.2 or 1.6 M), a non-ionic solute which cannot be metabolized by strain CHA0. For inoculation, aliquots of exponential-growth-phase KMB broth cultures of the bacterial strains were used to adjust the cell concentrations to an optical density at 600 nm (OD600) of 0.05. Cultures were incubated at 28±2°C with mild shaking. Bacterial growth was assessed 24 h after inoculation by plating serial dilutions of the cell suspensions on KMB. The experiment was repeated.

Effect of oxidative stress on bacteria

Sensitivity to paraquat (1,1'-dimethyl-4,4'-bipyridinium dichloride; Sigma), hydrogen peroxide (H2O2), or sodium hypochlorite (NaOCl) was examined as described by Martin *et al.* (1994). Filter disks (Whatman No. 1; diameter, 5 mm) were soaked with 20 μl of paraquat (1.9% w/v), H2O2 (3%, v/v), or NaOCl (5%, v/v) and placed on a layer of soft agar (2 ml of KMB with 0.8% agar) containing *P. fluorescens* overnight cultures. The diameters of the inhibition zones surrounding the impregnated disks were measured after overnight incubation at 28±2°C. The experiment was performed again.

Effect of thermal stress on bacteria

Wild type strain CHA0 and its mutant were grown at 30°C in 20 ml of KMB broth in 100-ml Erlenmeyer flasks. When the cultures reached an OD600 of 0.5, the flasks were incubated for 0, 15, 30, 60 and 120 min at 42 or 50°C. At each time point, three replicate cultures were sampled to determine the number of cfu on KMB agar plates. Colony counts were measured after incubation for 48 h at 28±2°C as mentioned above. The experiment was repeated.

Effect of pH stress on bacteria

For the pH stability test, the bacterium was cultivated at 28°C in 1/10th-strength KMB broth with a pH 3.0 (HCl) and 9.0 (NaOH). After 24 h, the bacteria were transferred back to 1/10th-strength KB broth with a normal pH of 5.7 and tested for colony counts. The experiment was performed again.

Effects of bacterial strains on *M. javanica* biocontrol in tomato

Sandy loam soil (pH 8.1, moisture retaining capacity of 38%) obtained from the experimental field of the Department of Botany, University of Karachi, was filled in 8 cm diam. plastic pots at 350 g pot^{-1}. Subsequently, a 25 ml cell suspension of CHA0 or CHA211 (2×10^8 cfu ml^{-1}) prepared in sterile $MgSO_4$ (0.1 M) was poured in the pots. Soil drenched with 25 ml sterile $MgSO_4$ (0.1 M) served as control. The procedure of bacterial cultivation and preparing of inoculum was the same as outlined above. After bacterial application, three tomato (*Lycopersicon esculentum* Mill.) seedlings were transplanted in each pot. One week after transplant, freshly hatched juvenile suspensions (< one-week-old) of *M. javanica* were inoculated in the soil. To introduce juveniles, 15 ml of a suspension containing 2000 juveniles was evenly distributed into four holes made 2 cm around the seedlings. Treatments and controls were replicated four times and randomized on a glasshouse bench. The soil was adjusted to 40% moisture holding capacity.

The experiment was terminated 45 days after the addition of nematodes and the numbers of galls induced by *M. javanica* on the entire root system were recorded. Plant growth parameters including fresh shoot and root weights were also measured. The root samples were divided into two equal portions. One of the portions of fresh roots was thoroughly washed with tap water, cut into small segments, reweighed, stained in acid fuchsine and homogenized in an electric grinder for 45 sec in water. The number of *M. javanica* juveniles that had penetrated the roots was counted and nematode populations on a per gram fresh root weight basis calculated. Rhizosphere populations of the two bacteria were isolated by placing root samples with adhering soil in a 250 ml Erlenmeyer flask containing 10 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 µl aliquots from the appropriate dilutions were plated onto KMB supplemented with tetracycline (50 mg l^{-1}). The plates were incubated at room temperature (28 ± 2°C) for 48 h and the number of cfu recorded.

Induced systemic resistance

The split-root (two-pot) system allows inoculation of the bacterium and nematode at separate locations on the root system (Siddiqui and Shaukat 2002). In this experiment, both sides of the root system were inoculated with *M. javanica* to determine whether control occurred when (i) the nematode was in direct contact with the bacteria and (ii) the nematode and bacteria were spatially separated which would indicate induced resistance.

Both halves of the root system were infested with 2000 freshly hatched juveniles of *M. javanica* while only one of the nematode-treated halves was inoculated with viable cell suspension of CHA0 or CHA211 (2×10^8 cfu ml^{-1}). Soil treated with sterile distilled water served as controls. Nematode penetration was determined 21 days after infestation from the nematode-treated half following the procedure outlined above. Populations of *P. fluorescens* in the rhizosphere were measured from non-bacterized half of the split root system. The experiment was repeated.

Statistical analysis

Data sets were subjected to analysis of variance using STATISTICA (ver. 5.0; Statsoft Incorp. Tulsa, Oklahoma, USA). When appropriate, means were separated with Fisher's protected ($P = 0.05$) least significant different (LSD) test or Duncan's multiple range test. Where the experiments were repeated, a Bartlett's test was performed to see if the treatment × trial interaction was significant. Since there was no significant difference among experiments, the data of the experiments was pooled. Bacterial population density data were normalized with a log10 plus 1 transformation prior to analysis.

RESULT

Protease production by *P. fluorescens* strains

Protease activity of the wild-type strain CHA0 resulted in clear zones (5 mm) formed around the growing colonies on skim milk agar. The mucoid mutant CHA211 showed a remarkably ($P < 0.05$) diminished zone (1 mm) of proteolytic activity on the growth medium.

Nematicidal activity of the bacteria

When compared to the controls, culture filtrate of the strain CHA0 obtained from 1/10-strength KMB resulted in mortality ($P < 0.05$) of *M. javanica* juveniles *in vitro* while strain CHA211 failed to produce nematicidal effects (data not presented).

Effect of osmotic stress on *P. fluorescens*

Compared to non-stressed conditions, osmotic concentrations resulting from NaCl or sorbitol reduced ($P < 0.05$) survival of *P. fluorescens* strains CHA0 and CHA211 (Table 1). Generally, both the bacterial strains were equally sensitive to osmotic concentrations, strain CHA211 exhibited greater ($P < 0.05$) survival compared to CHA0 when exposed to sorbitol at 1.2 M concentration.

Effect of oxidative stress on *P. fluorescens*

Pseudomonas fluorescens strain CHA0 was found highly sensitive to various oxidative stresses. For example, when filter paper disc impregnated with 1.9% (w/v) paraquat, 3% (v/v) H_2O_2 or 5% (v/v) NaOCl was placed on overnight cultures of *P. fluorescens*, zones of 14.5, 7.2 and 5.5 mm respectively, were produced around the colony of CHA0. Whereas strain CHA211 was as sensitive as CHA0 to paraquat and NaOCl, the mutant was more tolerant to H_2O_2 than its wild type counterpart (zone of inhibition of 7.2 was reduced to 4.5 mm).

Effect of thermal stress on *P. fluorescens*

Populations of the three bacterial strains declined ($P < 0.05$) consistently at both the temperatures (42 and 50°C) but the decline were steeper at 50°C (Fig.1). Strain CHA211 had significantly greater survival than strain CHA0 from 60 min onwards at 42°C and at all time periods when grown at 50°C.

Effect of pH stress on *P. fluorescens*

Regardless of strains, following transfer to acidic medium (pH 3.0) populations of both the bacteria were drastically reduced but subsequent to alkaline pH (9.0) exposure, the bacterial populations remained more or less unaltered compared to their growth at a usual pH of 5.7. Strain CHA211 showed the same level of sensitivity to acidic pH as did the wild type strain CHA0 (data not presented).

Effects of strain CHA0 and its mutants on *M. javanica* biocontrol in tomato

When compared to the controls, application of *P. fluorescens* strain CHA0 or CHA211 resulted in a significant ($P < 0.05$) reduction in nematode population densities in roots (Table 2). However, galling induced by *M. javanica* was markedly ($P < 0.05$) lower after treatment with strain CHA0 compared to the strain CHA211. None of the bacterial strains tested influenced root growth, strain CHA211 enhanced ($P < 0.05$) fresh weights of tomato seedlings. Rhizosphere colonization pattern of the bacterial inoculants was closely similar and that mucoidy in *P. fluorescens* did not improve bacterial survival.

P. fluorescens–mediated induction of systemic resistance against *M. javanica*

The application of root-knot nematode to both sides of a split root system followed by inoculation of bacterial inoculants to one side of nematode-infested section, significantly ($P < 0.05$) reduced nematode penetration in both sections of the roots compared to the controls (Fig. 2). However, regardless of the bacterial strains, nematode invasion was markedly lower in the root section where both nematode and bacteria were present. When efficacy of the inoculants was compared, wild type strain CHA0 caused greatest reduction of nematode invasion in both bacterized and non-bacterized halves of the split root system. Interestingly, expression of exopolysaccharides in the strain CHA211 reduced bacterial potential to induce systemic resistance in plants against root-knot nematode.

Table 1. The influence of osmotic stress on the survival of *Pseudomonas fluorescens* strain CHA0 and its mutant CHA211 *in vitro*.

Strains	Bacterial cfu log10(x+1)				
	Without Stress	NaCl (M) 0.6	0.8	Sorbitol (M) 1.2	1.6
CHA0	9.4a	7.3a	6.7a	5.1a	4.2a
CHA211	9.3a	7.6a	6.8a	5.7b	4.5a
LSD0.05	0.32	0.36	0.40	0.28	0.36

Means coincide with the same alphabet in each column are not significantly different ($P < 0.05$) as determined by the Duncan's multiple range test.

Table 2. The influence of *Pseudomonas fluorescens* strain CHA0 and its mucoid mutant CHA211 on nematode population densities in roots, gall development due to *Meloidogyne javanica* and growth of tomato seedlings.

Strains	*M. javanica* Juveniles/g root	Numbers of galls/g root	Shoot wt. (g)	Root wt. (g)	Bacterial colonization Log10 (x+1)
Control	212a	143a	3.1a	1.7a	-
CHA0	153c	104c	3.3a	1.7a	5.81a
CHA211	174bc	121ab	3.9a	2.1a	5.93a
LSD0.05	35	21	0.6	0.6	0.19

Means coincide with the same alphabet in each column are not significantly different ($P < 0.05$) as determined by the Duncan's multiple range test.

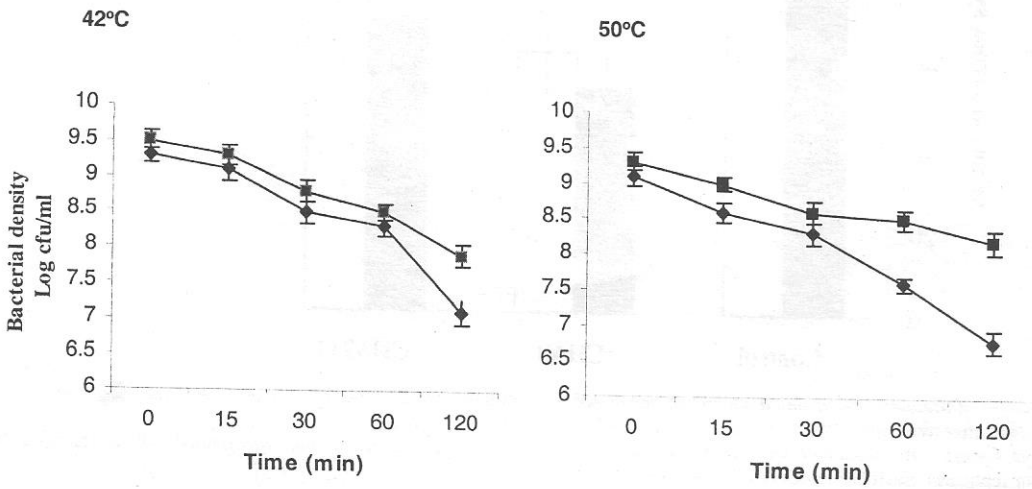

Fig. 1. The influence of thermal stress on density of *Pseudomonas fluorescens* strain CHA0 ◆ and its mucoid mutant CHA211 ■ at various time intervals *in vitro*. Each bar represents standard deviation; some of the error bars are too small to be distinguished.

DISCUSSION

The results of the present study imply that mucoidy in a mutant of *P. fluorescens* strain CHA0 imparts tolerance towards certain environmental stresses such as high osmolarity (sorbitol), oxidation (H_2O_2) and temperature (50°C) *in vitro*. Resistance of bacteria to specific stress conditions may be more crucial to survival in the rhizosphere than bulk soil due to an enhanced exposure to a specific stress factor in such environment (Miller and Wood, 1996). For example, among root-colonizing fluorescent *Pseudomonas* strains an apparent relation exists between colonization potential and *in vitro* osmotolerance (Loper *et al.*, 1985). Water metric potential is likely to be lower in the

rhizosphere of an actively transpiring plant than in the bulk soil, increases rhizosphere water osmolarity due to the exclusion of solutes by plant roots during water uptake. This attribute would likely provide a greater advantage to the mucoid mutants to survive on the rhizosphere than in bulk soil. The same argument could be proposed for oxidative stress tolerance. Colonization of plant root exudates would expose bacterial cells to root surface peroxidases that result in the production of superoxide anion and hydrogen peroxide (Albert *et al.,* 1986). Resistance to these oxidizing agents may facilitate root colonization by fluorescent pseudomonads (Katsuwon and Anderson, 1989). Exposure to high soil temperatures during hot summer season is another factor that limits survival and subsequent biological control potential of the inoculants. Antagonist with high thermotolerance would persist and proliferate for a longer period in such soils and contribute to an effective pathogen control. In this context, identification of the gene(s) responsible for enhanced stress tolerance in bacteria is a key to improve bacterial survival in the rhizosphere. Schnider-Keel *et al.,* (2001) demonstrated that *AlgU* is a second sigma factor, in addition to *RpoS* (Sarniguet *et al.,* 1995), involved in the response of *P. fluorescens* CHA0 to extreme environmental stress. Whereas strain CHA211 was more tolerant than wild type CHA0 against certain environmental stresses (i.e. high osmolarity, oxidation and temperature), the mutant failed to show enhanced tolerance towards paraquat, NaOCl, NaCl and acidic pH compared to wild type strain CHA0. Contrary to expectation this mutant failed to express defense-related functions despite EPS overproduction. One possibility is that *AlgU* acts as an on-off switch which, when activated (in the presence of a specific stress), drives the expression of stress defence-related genes (Schnider-Keel *et al.,* 2001).

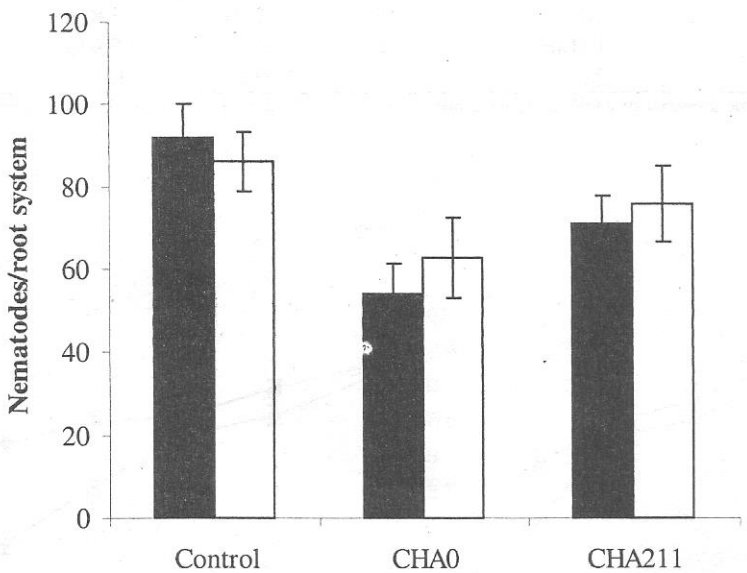

Fig. 2. Number of juveniles of *Meloidogyne javanica* which had penetrated the root in both bacterized ☐ and non-bacterized ■ root halves in a split root system 21 days after treatment with *P. fluorescens* CHA0, CHA211 or distilled water (control);
Both halves of the root system were inoculated with the nematodes while only one of the inoculated half was treated with the bacteria. Data represent means of four replicates. Each bar represents standard deviation.

The results of the present study indicate that whereas mucoidy in *P. fluorescens* strain CHA0 enhanced tolerance to specific stress conditions *in vitro*, our plant assays have failed to demonstrate any advantage for a mucoid mutant of strain CHA0 to colonize tomato rhizosphere. When colonization potential of the wild type strain CHA0 (non-mucoid) and strain CHA211 (a mucoid mutant of CHA0) was compared, no marked differences in their capacity to colonize the roots could be detected. It is possible that the (low) wild-type level of EPS production was already sufficient for maximum EPS-mediated stress protection of the bacterium in the rhizosphere and that stress defence may not be further optimized by EPS production in CHA211. Similar to our study, Schnider *et al.,* (1997), found no significant differences in the colonization pattern of the strains CHA0 and CHA211 in the rhizosphere and root surface of wheat.

In the present study, when nematicidal activity of the bacterial strains was compared, only strain CHA0 caused considerable mortality of *M. javanica* juveniles *in vitro*. Under glasshouse conditions, both the bacterial strains lowered *M. javanica* population densities in tomato roots but the magnitude of impact to control root-knot nematode

was lower for the strain CHA211. Production of secondary metabolites and extracellular proteases are considered to be a major mechanism involved in the control of soil-borne plant pathogens by fluorescent pseudomonads. Since strain CHA211 failed to produce extracellular proteases *in vitro*, it seems likely that biosynthesis of extracellular proteases by fluorescent pseudomonads are the important determinants for the suppression of plant parasitic nematodes. Furthermore, production of secondary metabolites such as diacetylphloroglucinol (Siddiqui and Shaukat, 2003), and hydrogen cyanide (Siddiqui *et al.*, unpubl.) by *P. fluorescens* CHA0 play a critical role in the suppression of root-knot nematode both *in vitro* and tomato roots. It is possible that diffusion of such metabolites into 1/10 strength KMB broth or the rhizosphere was restricted by the EPS layer over the growing colonies of CHA211 consequently reduced bacterial potential to control nematodes.

In this study, we demonstrated that mucoidy in *P. fluorescens* CHA0 did not improve bacterial efficacy to enhance plant defense mechanism in tomato roots against root-knot nematode. Reitz *et al.* (2000) have demonstrated that whereas purified EPS from *Rhizobium etli* strain G12 did not affect plant defense reactions in potato roots to infection by *Globodera pallida,* cyst nematode, and purified LPS impaired nematode infection by inducing systemic resistance. Since *R. etli* G12-mediated induced resistance is not associated with typical plant defense reactions such as enhanced activity of pathogenesis-related proteins or increased lignin content, even though the bacteria colonize the roots locally, Reitz *et al.* (2000) suggested that EPS of *R. etli* G12 may act as a suppressor of specific plant defense reactions. Results from the split root trial confirmed our previous finding that strain *Ps. fluorescens* CHA0 induces systemic resistance against root-knot nematode in tomato (Siddiqui and Shaukat, 2002) and that this strain suppresses nematode through a blend of mechanisms including secondary metabolite and enzyme production, and enhanced defence mechanism in plants leading to systemic resistance.

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