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Hydrogen Cyanide Produced by *Pseudomonas chlororaphis* O6 Exhibits Nematicidal Activity against *Meloidogyne hapla*

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Root-knot nematodes (*Meloidogyne* spp.) are parasites that attack many field crops and orchard trees, and affect both the quantity and quality of the products. A root-colonizing bacterium, *Pseudomonas chlororaphis* O6, possesses beneficial traits including strong nematicidal activity. To determine the molecular mechanisms involved in the nematicidal activity of *P. chlororaphis* O6, we constructed two mutants; one lacking hydrogen cyanide production, and a second lacking an insecticidal toxin, FitD. Root drenching with wild-type *P. chlororaphis* O6 cells caused juvenile mortality *in vitro* and *in planta*. Efficacy was not altered in the *fitD* mutant compared to the wild-type but was reduced in both bioassays for the mutant lacking hydrogen cyanide production. The reduced number of galls on tomato plants caused by the wild-type strain was comparable to that of a standard chemical nematicide. These findings suggest that hydrogen cyanide-producing root colonizers, such as *P. chlororaphis* O6, could be formulated as "green" nematicides that are compatible with many crops and offer agricultural sustainability.

Keywords **:** biological control, FitD insecticidal protein, hydrogen cyanide, nematicide, root-knot nematode

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Meloidogyne spp., cause serious damage to the roots of major crops including tomatoes, cucumbers, peppers, cabbages, sweet potatoes, and melons, with losses amounting to about 11% worldwide (McCarter, 2009). *Meloidogyne incognita*, *Meloidogyne javanica*, *Meloidogyne arenaria* and *Meloidogyne hapla* account for about 95% of this damage (Rich et al., 2009). In Korea, *M. incognita*, *M. arenaria*, and *M. hapla* are the major pests in certain vegetables, such as tomato (Choi and Choo, 1978; Rich et al., 2009). Based on field surveys performed in 1997-1999, *Meloidogyne* spp. infected about 54% of the sweet melon fields within Sungju province in Korea and resulted in yield losses (Cho et al., 2000; Kim, 2001). Cucumber yield was reduced by about 39% with a load exceeding 10 per 100 cm^3 conventional farming soil (Kim and Lee, 2008). Synthetic soil fumigants, such as methyl bromide, have been used aggressively to control nematodes, but their use is currently banned or restricted due to residual toxicity, and the need to conserve ecosystems (Abawi and Widmer, 2000). Soil solarization is an alternative, but costly control measure, both in time and effort (Kim et al., 2001). Consequently, safe and cost-effective methods for nematode control are required.

The development of "green" control formulations may be possible using nematicidal compounds produced by plants or microbes. Identified nematicidal microbial metabolites have different chemistries and include oxalic acid, which is produced by the fungus *Aspergillus niger* F22 (Jang et al., 2016), and 2,4-diacetylphloroglucinol (DAPG) from *Pseudomonas fluorescens* (Meyer et al., 2009; Siddiqui and Shaukat, 2003). Pyrrolnitrin and hydrogen cyanide (HCN) are implicated in the antagonistic activity of *P. chlororaphis* PA23 against the model nematode, *Caenorhabditis elegans* (Nandi et al., 2015). Consequently, control strategies may also involve formulations with microbes that are able to kill nematodes (Akhtar and Malik, 2000; Kerry, 2000;

Rodriguez-Kabana et al., 1987).

The biocontrol isolate, *P. chlororaphis* strain O6, is effective against the root knot nematode on tomato, and its production of HCN has been speculated to be a major inhibitor for nematicidal activity (Lee et al., 2011). Strain O6, like other pseudomonads, benefits plant growth through several mechanisms (Anderson and Kim, 2018). Root colonization by this isolate promotes plant growth and induces systemic resistance against various pathogens as well as drought and high salt abiotic stresses (Cho et al., 2008; Han et al., 2006; Kang et al., 2007; Kim et al., 2008). Metabolites including phenazines and pyrrolnitrin directly inhibit mycelial growth of plant pathogenic fungi (Kang et al., 2007; Park et al., 2011). *P. chlororaphis* O6 also possesses a *fit* (fluorescens insecticidal toxin) gene cluster, which regulates the synthesis of FitD, an insecticidal protein (Flury et al., 2016). Insecticidal activity is demonstrated for isolate O6 (Rangel et al., 2016), consistent with the oral toxicity of other pseudomonads that produce FitD (Péchy-Tarr et al., 2008).

The objective of this study was to confirm the protective role of HCN produced by *P. chlororaphis* O6 and to explore whether a cross-kingdom effect exists for FitD against the root knot nematode, *M. hapla.* The approach involved engineering mutants of isolate O6 lacking HCN or FitD production to probe the roles of these compounds in second juvenile stage (J2) mortality, which initiate infection of the host plant roots. The findings from this *in vitro* assay were compared to the results of an *in vivo* assay, in which changes in the number of galls formed on tomato due to root colonization by wild-type O6 cells were compared with the effects of the mutant strains. Another mutant, *gacS*, which lacks the global Gac/Rsm regulatory system (Anderson et al., 2017; Park et al., 2018), was also used to investigate how *P. chlororaphis* O6 regulates nematicidal products.

Materials and Methods

Construction of *P. chlororaphis* O6 *hcnA* and *fitD* mutants. General DNA manipulations, including gene cloning and plasmid isolation were performed using standard protocols (Sambrook et al., 1989). All restriction enzymes and modified enzymes were purchased from FastDigest, Thermo Scientic Korea Ltd (Seoul, Korea). The genome of *P. chlororaphis* O6 (Loper et al., 2012) contains both the *fit* gene cluster harboring *fitD* and the HCN biosynthesis operon, *hcnABC*. Specific primer sets were designed to amplify target genes. The *fitD* forward primer bearing a *Eco*R1 recognition site, 5’-CG**GAATTC**GGTTCTT-GTCGGCAAACCAC-3’ and the reverse primer harboring a *Hin*dIII site, 5’-CCC**AAGCTT**CCAATCACCTG-GTGTCGGAA-3’ encoded the amino acid sequences NHLVSE and WFADKNR within the FitD protein. The 4.3 kb PCR product contained a partial sequence of the *fitD* gene was cloned into the *Eco*RI- and *Hin*dIII-digested pEX18Tc marker exchange vector (Hoang et al., 1998).

To disrupt HCN production a forward primer was tagged with a *NsiI* site, 5’-CCA**ATGCAT**GCCTCTAT-GCCCTTCTGACC-3’ and the reverse primer was tagged with a *Sac*I site, 5’-TAC**GAGCTC**CAAGGCGTTTGCG-GAGTATG-3’. These primer sequences were designed to target sequences within the gene encoding a hypothetical protein located upstream and the gene for glutathione S-transferase located downstream of the *hcnABC* operon (Fig. 1). The 3.6 kb PCR product containing the *hcn* operon was digested with *Nsi*I and *Sac*I and cloned into the *Sac*I- and *Pst*I-digested pEX18Tc vector.

The pEX18Tc vectors containing the PCR products of the partial *fitD* and *hcn* operon genes were used for *in vitro* mutagenesis using EZ::Tn5 <KAN2> transposon (Epicentre Biotechnologies, WI, USA). This system randomly inserts a kanamycin resistance gene in the target DNA. The potential *fitD* or *hcn* mutants cloned into the pEX18Tc plasmid possessing tetracycline resistance were selected on LB agar plates containing tetracycline and kanamycin. The recombinant plasmids were prepared and used to determine the flanking sequence of the EZ-Tn5 transposon using the KAN-2 FP-1 forward primer and RP-1 reverse primers provided in the EZ::Tn5 <KAN2> transposon kit from Epicentre.

Two pEX18Tc recombinant clones, containing the EZ::Tn5 inserts within the structural genes of *fitD* and *hcnA* (Fig. 1), were selected and transferred into *P. chlororaphis* O6 through tri-parental mating. The marker-exchange mutant of each gene was selected on LB agar medium (Becton Dickinson GmbH, Heidelberg, Germany) supplemented with 5% sucrose and kanamycin in LB agar, as described previously (Miller et al., 1997). To confirm the mutation of both genes, PCR products were amplified from both mutants using gene specific primers, and the PCR products were sequenced to determine flanking sequences of EZ-Tn5 insertions in the mutants.

To complement the *hcn* mutant, the 3.6-kb PCR product containing the full-length *hcnABC* operon was cloned into pCRII vector, digested with *Nsi*I, and transferred into the *Pst*I-digested pCPP54 vector, which has a broad host range (Park et al., 2011). We could not complement the *fitD* mutant because this gene was embedded in the very large (about 12 kb) *fit* operon.

Culture conditions. The wild-type strain of *P. chlororaphis* O6, mutant strains defective in *gacS*, *hcnA*, and *fitD*, and the complemented *gacS* and *hcnA* mutants (Kang et al., 2007; Spencer et al., 2003) were used in the studies. Cells were grown to stationary phase for 36 h at 22°C with shaking at 200 rpm in liquid King's medium B (KB) (King et al., 1954), which was amended with glycine (4.5 g/l) to enhance HCN production. Glycine is the precursor of HCN (Zdor, 2015). *Escherichia coli* DH5α was cultivated for 48 h at 37°C with shaking at 250 rpm in the same media to provide a control bacterium not producing HCN or FitD.

Measurement of hydrogen cyanide production. *P. chlororaphis* O6 strains and *E. coli* DH5α, as a negative control, were grown in KB or KB plus glycine for 48 h at 22°C with agitation at 200 rpm in a shaking incubator. The cultures were centrifuged at 10,000 g to pellet the cells and the supernatants were filtered through 0.2 μm filters, and assayed to measure HCN (Guibault and Kramer, 1966). Dilutions (0.05 to 100 μm) of a KCN stock solution were used as the calibration standard.

To detect HCN production in the rhizosphere, tomato seeds (TENTEN, Koregon, Anseong, Korea) were surface sterilized with 70% ethanol for 5 min, treated with 1% sodium hypochlorite for 1 min and rinsed three times in sterile distilled water. Seeds were transferred to a sterile nursery soil mixture of Bio-Santo and Vermiculite (7:3, vol/vol, Seminis Korea, Seoul, Korea) contained in Magenta-boxes (7.2 × 7.2 × 10 cm, Sigma-Aldrich, St. Louis, MO, USA) and incubated for 16 h in light and 8 h in dark at 26°C. Two weeks after seeding, the roots were drenched with 10 ml of bacterial cells suspended in sterile water (1×10^8 cfu/ml) or with KB broth medium as a negative control. Three days after root drenching, HCN production was determined qualitatively using Cyantesmo paper (Machery-Nagel GmbH & Co., Duren, Germany).

***In vitro* nematicidal assay.** One bioassay for nematicidal activity was performed with the second juvenile stage (J2) of the root-knot nematode, *M. hapla*. The nematode juveniles were isolated from naturally infested soil at depths of 0–30 cm from the Fruit Experimental Station, in Haenam, Jeonnam, Korea (Lee et al., 2011). *P. chlororaphis* O6 bacterial strains were grown for 36 h in KB or KB plus glycine broth and adjusted to 1×10^8 cfu /ml (OD_{600nm} = 1.0) with sterile water, before preparing 10-fold dilutions in sterile water. The diluted cultures were mixed 1:1 v/v with a suspension of J2-stage juveniles (100 nematodes). After the mixtures were incubated for 1 h at room temperature, J2 mortality was evaluated under a low-power microscope (Carl Zeiss Discovery V12, Gottingen, Germany) by touching the nematodes with a sharp tip. Nematodes that did not respond with movement were considered dead. Assessment of each treatment involved three replicates examining responses with approximately 100 nematodes and three independent studies were conducted. Data represent the average of five replicates and the assay was repeated three times.

***In planta* nematicidal assay.** A second *in planta* nematicidal evaluation involved examining the effects on root-knot symptom formation on tomato following the methods modified by those of Lee et al (2011). Tomato seeds were surface-sterilized by soaking in 70% ethanol for 30 s and then 1% sodium hypochlorite for 10 min. After extensive washing with water, each tomato seed (Betatiny, PPS Seed, Yongin, Korea) was planted into sterile nursery soil mixture of Bio-Santo and Vermiculite housed in sterile Magenta boxes. Seedlings were established in closed Magenta boxes with lids. The lids were removed after development of the second true leaves of tomato plants, and 20 ml of sterile water was added as a drench every 2 days. The boxes were incubated in chambers under a regime of 16 h light (2,000 lux, 80 μmol photons m^{-2} s^{-1}) and 8 h darkness for 5 weeks.

The preventive and curative potential of the diluted bacterial cultures on the root knot symptoms were assessed. Cultures were grown on KB plus glycine as described above, and adjusted to 1×10^8 cfu/ml with sterile water. Each treatment box was drenched with 10 ml of the 10-fold diluted culture of the *P. chlororaphis* O6 strains. The nematicide, Fosthiazate 30% SL (FarmHannong, Seoul, Korea), was added to boxes as a positive treatment at the manufacturer's recommended dose (250 μl/l). Negative controls involved adding 10 ml of 10-fold diluted noninoculated KB plus glycine broth to the boxes. To assess the effects on nematode infection, the bacterial cultures were applied 1 week before nematode inoculation. To determine assess any curative effects, the bacterial cultures were added 1 week after nematode infection. Approximately 200 *M. hapla* J2 juveniles in 10 ml of sterile water were applied to each box. As a negative control, the boxes were drenched with the same volume of sterile water. The boxes were returned to the growth chamber under the same conditions as described above. After 2 weeks, the roots of plants were collected, and the numbers of root-knot galls were counted, and root fresh weights were recorded. The study was conducted with three replicates per treatment with three tomato plants per treatment.

Data analysis. Data were analyzed by ANOVA using SPSS 21.0 K for Windows software (SPSS institute, NC, USA). The significance of the effect of bacterial treatment was evaluated by Duncan's multiple range test ($P < 0.05$). The significance of the effect of glycine amendment in the KB medium of *P. chlororaphis* O6 strains was evaluated by Student's T-comparison ($P < 0.05$).

Results

Construction of *hcnA* or *fitD* mutants. Sequence analysis of PCR products obtained using DNA from the mutants confirmed the insertion of the EZ-transposon into the bacterial chromosome. The EZ-transposon disrupted the *hcnA* gene between the 16th amino acid, alanine, and the 17th amino acid, aspartic acid, relative to the start methionine. The selected *fitD* mutant contained an insertion between the 834th base pair in a codon encoding arginine, and the 835th base pair in a codon encoding tyrosine (Fig. 1). The altered genomes of the mutants were confirmed by PCR using the primer sets for each gene; an increase of about 1 kb in product size was observed as anticipated due to insertion of the kanamycin-resistance gene (Data not shown). The *fitD* mutant was additionally confirmed by transcriptional analysis between the wild-type and the *fitD* mutant (Supplementary Fig. 1). The *fitD* transcript was induced in the late-log phase and stationary phase in *P. chlororaphis* O6 wild-type, but no *fitD* transcripts were detected in the *fitD* mutant.

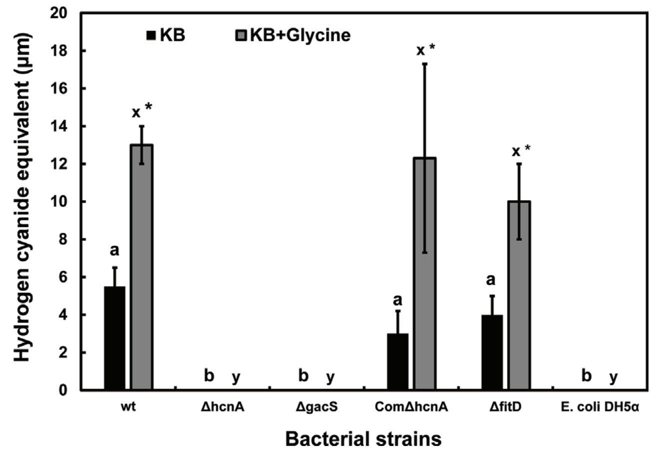

Fig. 2. *In vitro* hydrogen cyanide (HCN) production in *Pseudomonas chlororaphis* O6 wild-type and mutants. Two different growth media, KB medium and KB plus glycine, were used in the assessment of HCN production by the wild-type and mutants of *P. chlororaphis* O6. The procedures used to measure HCN are described in Materials and Methods. Strains used were: *P. chlororaphis* O6 wild-type (wt), the *hcnA* mutant (Δ*hcnA*), the *gacS* mutant (Δ*gacS*), the complemented-*hcnA* mutant (ComΔ*hcn*), the *fitD* mutant (Δ*fitD*), and as a negative control, *E. coli* DH5α. The data are expressed as the means with standard deviation of three replicates for the measurement of HCN. No HCN was detected with non-inoculated KB or KB plus glycine media (data not shown). Different letters indicate a statistically significant difference ($P < 0.05$) according to the results of Duncan's multiple test. *indicates a statistically significant difference ($P < 0.05$) based on Student's *t*-test upon glycine amendment in KB broth medium for each strain.

***In vitro* production of HCN.** Growth of the strains on KB broth with and without glycine amendments confirmed HCN production in the culture fluid at similar levels for the wild-type strain, the *fitD* mutant, and the complemented

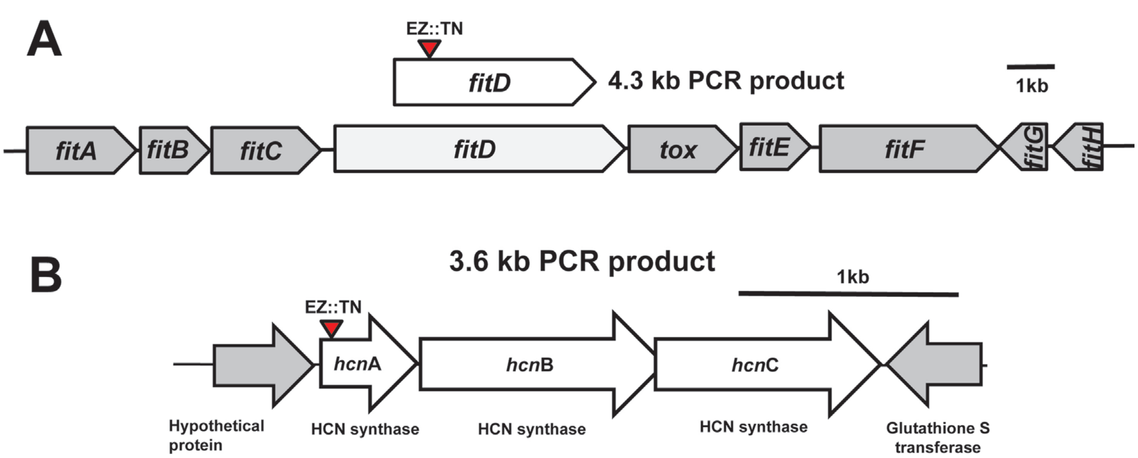

Fig. 1. *Pseudomonas chlororaphis* O6 gene sequences involved in HCN and FitD production. The *P. chlororaphis* O6 *hcnABC* operon and the *fit* operon, which contained the *fitD* gene encoding the insecticidal toxin (FitD) are shown. Arrows indicate the open reading frames and orientation of the genes. Vertical arrows indicated the insertion site of the EZ-TN cassette, which interrupted the *hcnA* and *fitD* genes.

hcn and *gacS* mutants (Fig. 2). These strains all presented increased HCN production when the medium was supplemented with the HCN-precursor, glycine (Fig. 2). However, no HCN production was observed following mutation of the *hcnA* gene in the HCN synthase operon and following mutation of the *gacS* gene. As anticipated, the control *E. coli* strain produced no HCN.

***In vitro* assay for J2 juvenile death.** Juvenile death was observed following application of intact cultures of the wild-type strain at 1×10^8, and to a lesser degree at 1×10^7 cfu/ml (Fig. 3). Higher mortality was observed when the wild-type strain was grown on KB plus glycine versus KB, supporting the involvement of HCN production in juvenile death. Similar levels of juvenile mortality were observed when the cultures were treated with the complemented *hcnA* mutant and the *fitD* mutant compared with the wild-type. Cultures with the *hcnA* mutant exhibited approximately 50% mortality, similar to the level (53 to 60% mortality) observed with the *gacS* mutant, which does not produce HCN. No nematicidal activity of *E. coli* DH5α and KB medium was observed during the initial inoculation. However, after 1 h of treatment with *E. coli* DH5α cultures presented 20-30% loss in viability; these values are similar to that observed with non-inoculated KB medium.

Effects of bacterial cells and metabolites on gall formation in tomato. In this study, all bacterial cultures were grown on KB plus glycine broth to optimize HCN formation. No galls were observed on tomato roots grown in the absence of *M. hapla* infection (Fig. 4). The plant infective J2 juveniles were used as the inoculum and galls were assessed in 14 day-old plants. Under the preventative regime, when nematodes and bacterial preparations were applied at the same time, and application of the nematicide, Fosthiazate, eliminated gall formation. Gall numbers also decreased from about 100 galls/plant in the nematode control study to about 40 galls/plant with treatments of the wild-type cultures or the preparations from the complemented *hcn* mutant and the *fitD* mutant. There was no significant difference in the gall numbers between the no-treatment control and treatment of the *hcn* mutant bacterial preparations.

To examine a curative effect, nematode inoculation was

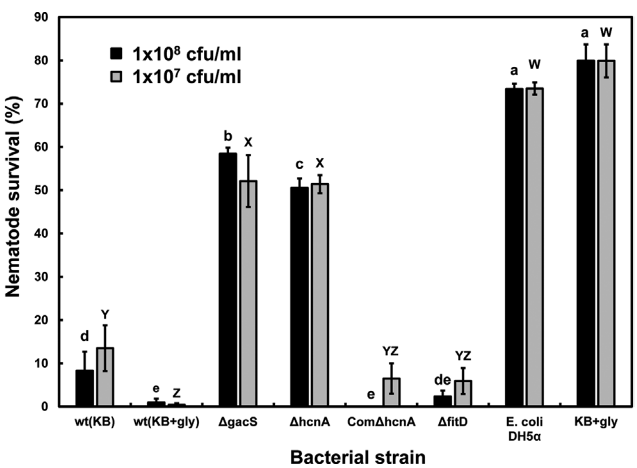

Fig. 3. *In vitro* nematicidal effect of bacterial cultures of *Pseudomonas chlororaphis* O6 wild-type and mutant strains on the second stage juveniles of the root-knot nematode, *Meloidogyne hapla*. Two different growth media, KB medium and KB plus glycine, were used to evaluate the effects of the mixture of bacterial cells and their metabolites on the mortality of the infective juvenile stage of the root-knot nematode. Cultures were generated from: *P. chlororaphis* O6 wild-type (wt), the *hcnA* mutant (Δ*hcnA*), the *gacS* mutant (Δ*gacS*), the complemented-*hcn* mutant (ComΔ*hcn*), the *fitD* mutant (Δ*fitD*), and *E. coli* DH5α as a negative control. Approximately 100 J2 juveniles were used for each experiment. Data are expressed as the percent mean nematode survival after microbial treatment compared with the controls. The standard deviations shown are based on three replicates. Different letters indicate a statistically significant difference ($P < 0.05$) by Duncan's multiple test.

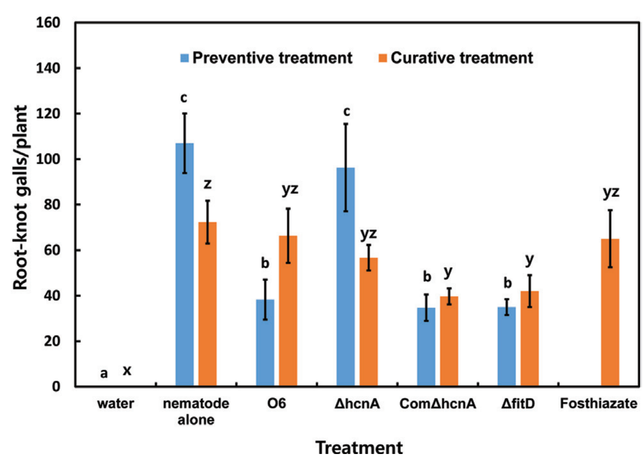

Fig. 4. Reduction in galls in tomato plants. Nematicidal activities of bacterial cultures of *Pseudomonas chlororaphis* O6 strains were examined based on gall number with procedures to assess both preventive and curative effects as described in Materials and Methods. Approximately 500 J2 juveniles of *Meloidogyne hapla* in 10 ml of sterile water were used per box as inoculum and 10 ml of sterile water was used as a negative control (water). Three replicates were used for each treatment, with three tomato plants each treatment. Data are expressed as the mean and standard deviation of three replicates. Different letters indicate a statistically significant difference between treatments ($P < 0.05$) by Duncan's multiple test.

performed 1 week prior to the addition of bacterial preparations or the commercial nematicide. The gall numbers were lower with the 7 day-delayed applications compared with the preventive regime for the control (Fig. 4). No curative effects were observed for the treatments with the wild-type strain, the *hcn* mutant, and Fosthiazate. However, there was a trend for lower gall numbers in treatments with the complemented *hcn* mutant and the *fitD* mutant.

Tomato root colonization and HCN production in the rhizosphere. To establish whether the loss of protection against gall formation by the *hcn* mutant was due to a lack of root colonization, colonization of this mutant was compared with that of the wild-type strain. The same number of cells was recovered from the tomato roots in the wild-type and mutant when assayed at 3, 5 and 7 days after inoculation (Supplementary Fig. 2). The use of Cyantesmo paper in the air space of the plant growth boxes confirmed HCN production, as indicated by blue coloration of the indicator paper, only when the tomato roots were colonized by the wild-type cells. As anticipated, no coloration was detected when roots were colonized by the *hcnA* or *gacS* mutants (Supplementary Fig. 3).

Discussion

Control of *M. hapla* by the wild-type *P. chlororaphis* O6 strain, observed as increases in juvenile motality and decreases in gall formation in tomato roots, confirmed our previous findings (Lee et al., 2011). The toxic effect of HCN on nematodes is consistent with the loss of mitochondrial function through the inhibition of cytochrome c oxidase (Zdor, 2015). Another factor could be the sequestration of Fe from the host cells due to the formation of FeCN (Rijavec and Lapanje, 2016).

Mutation of the *fit*D gene did not reduce the mutant's ability to kill nematode juveniles consistent with HCN production by the wild-type. These findings indicate that the insect toxin FitD is not a major factor in cross-kingdom nematode protection. *P. chlororaphis* O6 is among other pseudomonads that were lethal to the horn worm larvae when injected or given orally due to the expression of *fitD* (Rangel et al., 2016).

The reduction of juvenile mortality with the *hcnA* mutant, and restoration of this trait by complementation with the complete *hcn* operon, suggested a major role of HCN-induced damage for the root knot nematode. The observation that a *gacS* mutant induced juvenile mortality to a similar extent as the single *hcnA* mutant under the bioassay conditions indicated that the loss of HCN, rather than other factors in the Gac/Rsm regulatory pathway, was involved in nematode lethality. Similarly, the reduced number of root knot galls in tomato plants when treated with the wild-type or the complemented *hcnA* mutant, but not with the *hcnA* mutant, confirmed a major role of HCN in the control of gall formation. This could be a consequence of juvenile death resulting in lesser infection, and/or the systemic activation of plant defense pathways by the bacterium effective against gall formation (Spencer et al., 2003). The concept that reduced gall formation in the plant is due to the death of juveniles is supported by the limited curative protection by the treatments.

Growth on KB plus glycine boosted HCN production in the wild-type, which is consistent with the known pathway of HCN production from glycine by a synthase composed of three subunits encoded by the *hcn*ABC operon (Zdor, 2015). Glycine is present in the root exudates of many plants and is found in soil pore waters (Fischer et al., 2007; Kamilova et al., 2006; Lesuffleur et al., 2007). Thus, external sources of glycine are available for the production of HCN by microbes, such as *P. chlororaphis* O6, in the rhizosphere. HCN was present in the air space when tomato was grown with roots colonized by the wild-type strain, but not with those colonized by the *hcnA* or *gacS* mutants.

HCN production from the *hcnABC* operon in *P. aeruginosa* is regulated by the Gac/Rsm system, consistent with the maximum level observed at high cell density (Lapouge et al., 2008). Additionally, it is also controlled by an anerobic sensor system, with ANR (anaerobic regulator of arginine deiminase and nitrate reductase) acting as the regulator (Pessi and Haas, 2000). These conditions correlate with biofilm-containing clustes of *P. chlororaphis* O6 cells at the root surface, which display limited fermentative metabolism (Wright et al., 2016).

Both HCN and pyrrolnitrin from *P. chlororaphis* PA23 are reportedly involved in cell death of the model nematode *C. elegans* (Nandi et al., 2015). Curiously pyrrolnitrin, also a metabolite of *P. chlororaphis* O6, inhibits HCN production in cells of another pseudomonad, perhaps due to the effects on the glycine dehydrogenase, which is involved in HCN synthesis (Wissing, 1974). The toxic effect of pyrrolnitrin is correlated with the inhibition of electron transport (Tripathi and Gottlieb, 1969; Wong and Airall, 1970). The extent to which such interactions occur in the rhizosphere has not been determined. It is possible that *C. elegans* and *M. hapla* differ in their responsiveness to pyrrolnitrin. Additionally, it is possible that when examing root-gall formation on tomato during the *in planta* assays, pyrrrolnitrin was not produced by the O6 strain at sufficiently high concentrations to inhibit nematode activity.

The finding that both the *gacS* and the *hcnA* mutant retained the ability to induce larval mortality, could be explained by a role of the fluorescent siderophore in nematode death. Both these mutants produce the fluorescent siderophore, pyoverdine, which disturbs Fe homeostasis in the host nematode. This possibility will be further explored by examining the activity in *P. chlororaphis* O6 mutants lacking in pyoverdine formation.

These findings illustrate the breadth of the cross-kingdom influence of the biocontrol strain *P. chlororaphis* O6, which is potentially active in the rhizosphere. It is important to understand how to adjust the metabolism of beneficial root-colonizing microbes in order to enhance the control of pathogens and pests under field conditions. Glycine is the precursor for HCN; this suggests that glycine could be added to formulations of bacterial cultures with *hcn* operons to provide increased availability of substrate. Controlled production of active pesticidal metabolites in the rhizosphere could enhance agricultural sustainability and crop yield.

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Conflict of Interest

The authors declare that they have no competing and commercial interests in his work.

References

Abawi, G. S. and Widmer, T. L. 2000. Impact of soil health management practices on soilborne pathogens, nematodes and root diseases of vegetable crops. *Appl. Soil Ecol.* 15:37-47.

Akhtar, M. and Malik, A. 2000. Roles of organic soil amendments and soil organisms in the biological control of plant-parasitic nematodes: a review. *Bioresour. Technol.* 74:35-47.

Anderson, A. J., Kang, B. R. and Kim, Y. C. 2017. The Gac/Rsm signaling pathway of a biocontrol bacterium, *Pseudomonas chlororaphis* O6. *Res. Plant Dis.* 23:212-227.

Anderson, A. J. and Kim, Y. C. 2018. Biopesticides produced by plant-probiotic *Pseudomonas chlororaphis* isolates. *Crop Prot.* 105:62-69.

Cho, M. R., Lee, B. C., Kim, D. S., Jeon, H. Y., Yiem, M. S. and Lee, J. O. 2000. Distribution of plant-parasitic nematodes in fruit vegetable production areas in Korea and identification of root-knot nematodes by enzyme phenotypes. *Korean J. Appl. Entomol.* 39:123-129.

Cho, S. M., Kang, B. R., Han, S. H., Anderson, A. J., Park, J. Y., Lee, Y. H., Cho, B. H., Yang, K. Y., Ryu, C. M. and Kim, Y. C. 2008. 2R,3R-butanediol, a bacterial volatile produced by *Pseudomonas chlororaphis* O6, is involved in induction of systemic tolerance to drought in *Arabidopsis thaliana*. *Mol. Plant-Microbe Interact.* 21:1067-1075.

Choi, Y. E. and Choo, H. Y. 1978. A study on the root-knot nematodes (*Meloidogyne* spp.) affecting economic crops in Korea. *Korean J. Appl. Entomol.* 17:89-98.

Fischer, H., Meyer, A., Fischer, K. and Kuzyakov, Y. 2007. Carbohydrate and amino acid composition of dissolved organic matter leached from soil. *Soil Biol. Biochem.* 39:2926-2935.

Flury, P., Aellen, N., Ruffner, B., Pechy-Tarr, M., Fataar, S., Metla, Z., Dominguez-Ferreras, A., Bloemberg, G., Frey, J., Goesmann, A., Raaijmakers, J. M., Duffy, B., Hofte, M., Blom, J., Smits, T. H., Keel, C. and Maurhofer, M. 2016. Insect pathogenicity in plant-beneficial pseudomonads: phylogenetic distribution and comparative genomics. *ISME J.* 10: 2527-2542.

Guibault, G. G. and Kramer, D. N. 1966. Ultra sensitive, specific method for cyanide using p-nitrobenzaldehyde and o-dinitrobenzene. *Anal. Chem.* 38:834-836.

Han, S. H., Lee, S. J., Moon, J. H., Park, K. H., Yang, K. Y., Cho, B. H., Kim, K. Y., Kim, Y. W., Lee, M. C., Anderson, A. J. and Kim, Y. C. 2006. GacS-dependent production of 2R, 3R-butanediol by *Pseudomonas chlororaphis* O6 is a major determinant for eliciting systemic resistance against *Erwinia carotovora* but not against *Pseudomonas syringae* pv. *tabaci* in tobacco. *Mol. Plant-Microbe Interact.* 19:924-930.

Hoang, T. T., Karkhoff-Schweizer, R. R., Kutchma, A. J. and Schweizer, H. P. 1998. A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. *Gene* 212:77-86.

Jang, J. Y., Choi, Y. H., Shin, T. S., Kim, T. H., Shin, K.-S., Park, H. W., Kim, Y. H., Kim, H., Choi, G. J. and Jang, K. S. 2016. Biological control of *Meloidogyne incognita* by *Aspergillus niger* F22 producing oxalic acid. *PLoS One* 11:e0156230.

Kamilova, F., Kravchenko, L. V., Shaposhnikov, A. I., Azarova, T., Makarova, N. and Lugtenberg, B. 2006. Organic acids, sugars, and L-tryptophane in exudates of vegetables growing on stonewool and their effects on activities of rhizosphere bacteria. *Mol. Plant-Microbe Interact.* 19:250-256.

Kang, B. R., Han, S.-H., Zdor, R. E., Anderson, A. J., Spencer, M., Yang, K. Y., Kim, Y. H., Lee, M. C., Cho, B. H. and Kim, Y. C. 2007. Inhibition of seed germination and induction of systemic disease resistance by *Pseudomonas chlororaphis* O6 requires phenazine production regulated by the global regulator, *gacS*. *J. Microbiol. Biotech.* 17:586-593.

Kerry, B. R. 2000. Rhizosphere interactions and the exploitation of microbial agents for the biological control of plant-parasitic nematodes. *Annu. Rev. Phytopathol.* 38:423-441.

Kim, D.-G. and Lee, J.-H. 2008. Economic threshold of *Meloidogyne incognita* for greenhouse grown cucumber in Korea. *Res. Plant Dis.* 14:117-121.

Kim, D. G. 2001. Occurrence of root-knot nematodes on fruit vegetables under greenhouse conditions in Korea. *Res. Plant Dis.* 7:69-79.

Kim, D. G., Choi, D. R. and Lee, S. B. 2001. Effects of control methods on yields of oriental melon in fields infested with *Meloidogyne arenaria*. *Res. Plant Dis.* 7:42-48.

Kim, M. S., Cho, S. M., Kang, E. Y., Im, Y. J., Hwangbo, H., Kim, Y. C., Ryu, C. M., Yang, K. Y., Chung, G. C. and Cho, B. H. 2008. Galactinol is a signaling component of the induced systemic resistance caused by *Pseudomonas chlororaphis* O6 root colonization. *Mol. Plant-Microbe Interact.* 21:1643-1653.

King, E. O., Ward, M. and Raney, D. 1954. Two simple media for the demonstration of pyocyanin and fluorescein. *J. Lab. Clin. Med.* 44:301-307.

Lapouge, K., Schubert, M., Allain, F. H. and Haas, D. 2008. Gac/Rsm signal transduction pathway of γ-proteobacteria: from RNA recognition to regulation of social behavior. *Mol. Microbiol.* 67:241-253.

Lee, J. H., Ma, K. C., Ko, S. J., Kang, B. R., Kim, I. S. and Kim, Y. C. 2011. Nematicidal activity of a nonpathogenic biocontrol bacterium, *Pseudomonas chlororaphis* O6. *Curr. Microbiol.* 62:746-751.

Lesuffleur, F., Paynel, F., Bataillé, M.-P., Le Deunff, E. and Cliquet, J.-B. 2007. Root amino acid exudation: measurement of high efflux rates of glycine and serine from six different plant species. *Plant Soil* 294:235-246.

Loper, J. E., Hassan, K. A., Mavrodi, D. V., Davis, E. W., Lim, C. K., Shaffer, B. T., Elbourne, L. D. H., Stockwell, V. O., Hartney, S. L., Breakwell, K., Henkels, M. D., Tetu, S. G., Rangel, L. I., Kidarsa, T. A., Wilson, N. L., de Mortel, J. E. V., Song, C. X., Blumhagen, R., Radune, D., Hostetler, J. B., Brinkac, L. M., Durkin, A. S., Kluepfel, D. A., Wechter, W. P., Anderson, A. J., Kim, Y. C., Pierson, L. S., Pierson, E. A., Lindow, S. E., Kobayashi, D. Y., Raaijmakers, J. M., Weller, D. M., Thomashow, L. S., Allen, A. E. and Paulsen, I. T. 2012. Comparative genomics of plant-associated Pseudomonas spp.: Insights into diversity and inheritance of traits involved in multitrophic interactions. *PLoS Genet.* 8:e1002784.

McCarter, J. 2009. Molecular approaches toward resistance to plant-parasitic nematodes. In: *Cell biology of plant nematode parasitism*, pp. 239-267. Springer.

Meyer, S. L. F., Halbrendt, J. M., Carta, L. K., Skantar, A. M., Liu, T., Abdelnabby, H. M. E. and Vinyard, B. T. 2009. Toxicity of 2,4-diacetylphloroglucinol (DAPG) to plant-parasitic and bacterial-feeding nematodes. *J. Nematol.* 41:274-280.

Miller, C. D., Kim, Y. C. and Anderson, A. J. 1997. Cloning and mutational analysis of the gene for the stationary-phase inducible catalase (*catC*) from *Pseudomonas putida*. *J. Bacteriol.* 179:5241-5245.

Nandi, M., Selin, C., Brassinga, A. K. C., Belmonte, M. F., Fernando, W. D., Loewen, P. C. and De Kievit, T. R. 2015. Pyrrolnitrin and hydrogen cyanide production by *Pseudomonas chlororaphis* strain PA23 exhibits nematicidal and repellent activity against *Caenorhabditis elegans*. *PLoS One* 10:e0123184.

Park, J. Y., Kang, B. R., Ryu, C.-M., Anderson, A. J. and Kim, Y. C. 2018. Polyamine is a critical determinant of *Pseudomonas chlororaphis* O6 for GacS-depedent bacterial cell growth and biocontrol activity. *Mol. Plant Pathol*. doi: 10.1111/mpp.12610 (in press).

Park, J. Y., Oh, S. A., Anderson, A. J., Neiswender, J., Kim, J. C. and Kim, Y. C. 2011. Production of the antifungal compounds phenazine and pyrrolnitrin from *Pseudomonas chlororaphis* O6 is differentially regulated by glucose. *Lett. Appl. Microbiol.* 52:532-537.

Péchy-Tarr, M., Bruck, D. J., Maurhofer, M., Fischer, E., Vogne, C., Henkels, M. D., Donahue, K. M., Grunder, J., Loper, J. E. and Keel, C. 2008. Molecular analysis of a novel gene cluster encoding an insect toxin in plant-associated strains of *Pseudomonas fluorescens*. *Environ. Microbiol.* 10:2368-2386.

Pessi, G. and Haas, D. 2000. Transcriptional control of the hydrogen cyanide biosynthetic genes *hcnABC* by the anaerobic regulator ANR and the quorum-sensing regulators LasR and RhlR in *Pseudomonas aeruginosa*. *J. Bacteriol.* 182:6940-6949.

Rangel, L. I., Henkels, M. D., Shaffer, B. T., Walker, F. L., Davis II, E. W., Stockwell, V. O., Bruck, D., Taylor, B. J. and Loper, J. E. 2016. Characterization of toxin complex gene clusters and insect toxicity of bacteria representing four subgroups of *Pseudomonas fluorescens*. *PLoS One* 11:e0161120.

Rich, J. R., Brito, J. A., Kaur, R. and Ferrell, J. A. 2009. Weed species as hosts of *Meloidogyne*: a review. *Nematropica* 39:157-185.

Rijavec, T. and Lapanje, A. 2016. Hydrogen cyanide in the rhizosphere: not suppressing plant pathogens, but rather regulating availability of phosphate. *Front. Microbiol.* 7:1785.

Rodriguez-Kabana, R., Morgan-Jones, G. and Chet, I. 1987. Biological control of nematodes: Soil amendments and microbial antagonists. *Plant Soil* 100:237-247.

Sambrook, J. 2001. Sambrook, J., Fritsch, E. F. and Maniatis, T. 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, NY, USA.

Siddiqui, I. A. and Shaukat, S. S. 2003. Suppression of root-knot disease by *Pseudomonas fluorescens* CHA0 in tomato: importance of bacterial secondary metabolite, 2,4-diacetylpholoroglucinol. *Soil Biol. Biochem.* 35:1615-1623.

Spencer, M., Ryu, C. M., Yang, K. Y., Kim, Y. C., Kloepper, J. W. and Anderson, A. J. 2003. Induced defence in tobacco by *Pseudomonas chlororaphis* strain O6 involves at least the ethylene pathway. *Physiol. Mol. Plant Pathol.* 63:27-34.

Tripathi, R. K. and Gottlieb, D. 1969. Mechanism of action of the antifungal antibiotic pyrrolnitrin. *J. Bacteriol.* 100:310-318.

Wissing, F. 1974. Cyanide formation from oxidation of glycine of

Pseudomonas species. *J. Bacteriol.* 117:1289-1294.

Wong, D. T. and Airall, J. M. 1970. The mode of action of antifungal agents: effect of pyrrolnitrin on mitochondrial electron transport. *J. Antibiot.* 23:55-62.

Wright, M., Adams, J., Yang, K., McManus, P., Jacobson, A., Gade, A., McLean, J., Britt, D. and Anderson, A. 2016. A root-colonizing pseudomonad lessens stress responses in wheat imposed by CuO nanoparticles. *PLoS One* 11:e0164635.

Zdor, R. E. 2015. Bacterial cyanogenesis: impact on biotic interactions. *J. Appl. Microbiol.* 118:267-274.