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Inhibition of Seed Germination and Induction of Systemic Disease Resistance by *Pseudomonas chlororaphis* O6 Requires Phenazine Production Regulated by the Global Regulator, GacS

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Abstract Seed coating by a phenazine-producing bacterium, *Pseudomonas chlororaphis* O6, induced dose-dependent inhibition of germination in wheat and barley seeds, but did not inhibit germination of rice or cucumber seeds. In wheat seedlings grown from inoculated seeds, phenazine production levels near the seed were higher than in the roots. Deletion of the *gacS* gene reduced transcription from the genes required for phenazine synthesis, the regulatory *phzI* gene and the biosynthetic *phzA* gene. The inhibition of seed germination and the induction of systemic disease resistance against a bacterial soft-rot pathogen, *Erwinia carotovora* subsp. *carotovora*, were impaired in the *gacS* and *phzA* mutants of *P. chlororaphis* O6. Culture filtrates of the *gacS* and *phzA* mutants of *P. chlororaphis* O6 did not inhibit seed germination of wheat, whereas that of the wild-type was inhibitory. Our results showed that the production of phenazines by *P. chlororaphis* O6 was correlated with reduced germination of barley and wheat seeds, and the level of systemic resistance in tobacco against *E. carotovora*.

Keywords: GacS/GacA two-component system, induced systemic resistance, phenazine, inhibition of seed germination

Certain rhizosphere microbes stimulate plant growth and suppress plant pathogens via the production of secondary metabolites that inhibit the growth of phytopathogenic microbes [3, 9, 11, 13, 20, 29]. *Pseudomonas chlororaphis* O6, a nonpathogenic root-colonizing bacterium, produces both phenazine and hydrogen cyanide [18, 22, 26]. When tested on King's medium B [16] that supports the production of phenazines, the wild-type strain inhibits the growth of the wheat fungal pathogen *Fusarium culmorum* [22, 26]. However, in field studies performed to characterize the potential biocontrol activity of this bacterium against *F. culmorum*, the treatment of seeds with *P. chlororaphis* O6 reduced the emergence of wheat seeds [18]. Phytotoxicity has been attributed to the production of HCN by rhizosphere pseudomonads in potatoes [3, 24], lettuce, barnyard grass [17], and velvet leaf [8]. Furthermore, the production of phenazine-1-carboxylic acid has been implicated in the phytotoxicity of *Pseudomonas fluorescens* strain 2-79 on the germination of treated wheat seeds [25].

Hydrogen cyanide and phenazine production in the pseudomonads requires the expression of biosynthetic genes present in the *hcn* and *phz* operons. The regulation of expression of the *phz* biosynthetic genes requires upstream consensus sequences, termed Lux or Phz boxes depending on whether the bacterium is a *Vibrio* or a phenazine-producing pseudomonad, and they function as sites in which transcriptional activators interact with the quorum-sensing molecules, *N*-acyl-homoserine lactones (AHSLs) [9, 27]. AHSL production is controlled by a two-component system, GacS/GacA [5, 12, 28]: phosphotransfer from the activated plasma membrane sensor kinase, GacS, to the response regulator, GacA, initiates AHSL signaling [12].

The colonization of roots by *P. chlororaphis* O6 induces systemic protection; for example, against the leaf pathogen *Corynespora cassicola* in cucumber [15] and against the soft-rot pathogen *Erwinia carotovora* in tobacco [26]. Induced

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resistance against *E. carotovora* requires a functional *gacS* gene in the *P. chlororaphis* O6 cells colonizing roots [26].

This study was undertaken to examine the roles of phenazines and hydrogen cyanide production in *P. chlororaphis* O6 in the inhibition of seed germination. We first examined this isolate for homologs of the regulatory gene, *phzI*, and the biosynthesis genes, *phzAB*, detected in other phenazine-producing pseudomonads [28]. The transcriptional regulation of the *phzI* and *phzA* genes in the wild-type and in the *gacS* mutant was assessed to gain further insight into the regulatory role of GacS in their expression. Mutants of *phzA* were prepared to elucidate the role of phenazine production in seed germination and to examine the effect of phenazine production on induced resistance against the soft-rot pathogen in tobacco.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Bacteria were stored in 15% glycerol at −70°C. A *gacS* mutant and a *gacS*-complemented strain of *P. chlororaphis* O6 were constructed as described by Spencer *et al.* [26]. The *phz* mutants and wild-type pseudomonad cells were grown at 28°C with agitation at 200 rpm in King's medium B (KB) [16] broth for routine culturing, and in Luria-Bertani (LB) agar for colony counting. The cells utilized for seed inoculation were grown to an optical density of 2.0 at 600 nm in KB broth, centrifuged, and resuspended to desired concentrations in sterile 50 mM potassium phosphate buffer (pH 7.5). When appropriate, the bacteria were cultured in media supplemented with antibiotics: 100 μg/ml ampicillin (Ap), 50 μg/ml kanamycin (Km) (the *gacS* mutant), and 20 μg/ml tetracycline (Tc) for the complemented *gacS* mutants.

DNA Manipulations

DNA manipulations for cloning and subcloning, including transformations, restriction enzyme digestions, ligations, and electrophoresis, were conducted according to the methods described by Ausubel *et al.* [2] and Sambrook and Russell [23]. Chromosomal DNA was prepared by the hexadecyltrimethylammonium bromide (CTAB) method [2]. Southern or colony hybridizations were conducted using the nonradioactive Genius system (Roche Molecular Biochemicals, Mannheim, Germany). Nucleotide sequence analyses were conducted using a dye terminator on an ABI1301 DNA sequencer (Applied Biosystems, Foster City, CA, U.S.A.) at the Gwangju branch of the Korea Basic Science Institute (KBSI).

Cloning of the *phz* Operon

To examine the genes involved in phenazine production in *P. chlororaphis* O6, primers were designed from *P. chlororaphis* PCL1391. The primers were as follows: for *phzI*, forward, 5'-GC(T)T TTC GGC AC(T)G AAC AA(G)T TC-3'; reverse, 5'-CCC AGG(T) CGT TCG TAG(C) TG(T)G AC-3') and *phzAB*, forward, 5'-CAT GAA CG(C)C(T) G(C)TT C(T)G(C)A A(G)CA AC(T)T-3'; reverse, 5'-CGA CCC AGA A(G,T)A(G)T GA(G)T TA(C,G)G GA(G)T C-3'. The PCR reaction was conducted with pre-mix (Bioneer Inc., Daejeon, Republic of Korea) in the GenAmp 2400 thermal cycler (Perkin Elmer, San Jose, CA, U.S.A.). The *phz* gene PCR products were generated from *P. chlororaphis* O6 genomic DNA, and the products, 384 bp for *phzI* and 395 bp for *phzAB*, were sequenced. The PCR products were used to probe the genomic library of *P. chlororaphis* O6, which was constructed previously using the broad host range cosmid vector pCPP47. A positive clone, the pOphzI plasmid, was subcloned to yield a 14-kb EcoRI fragment (pPOE14) harboring the phenazine biosynthetic genes. Further subcloning was carried out to determine the complete nucleotide sequence of the phenazine biosynthetic operon of *P. chlororaphis* O6. These nucleotide sequences were deposited in the GenBank database (NCBI, http://www.ncbi.nlm.nih.gov) under the accession number AY927995.

Transcriptional Analysis by Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Cells grown in KB broth were harvested in mid-logarithmic phase (OD 600 nm=0.8), late-logarithmic phase (OD 600 nm =1.8), and stationary phase (OD 600 nm=2.4). Total RNA was isolated via the Trizol method, according to the recommendations of the manufacturer (GIBCO BRL, Gaitherburg, MD, U.S.A.). RT-PCR was conducted with the QuantiTect SYBR Green reverse transcription-PCR kit (Quiagen Cat. No. 204243, Valencia, CA, U.S.A.) as previously described [14]. The PCR primers employed in this study were as follows: *gacS* (forward, 5'-GCA AAC CCA ACT TCT GCA AC-3'; reverse, 5'-GTC CTG CTG TTC CAG GGA CT-3'), *phzI* (forward, 5'-CAC ACT GAG CGA AAT GGA C-3'; reverse, 5'-CCA TTC ACA AAC TGC CTG-3'), *phzA* (forward, 5'-CCT AGC GGC TTT AAT GAT C-3'; reverse, 5'-GCC ATC CTG AGT GAA CAG-3'), and 16S rRNA (forward, 5'-TGG CTC AGA ACG AAC GCT GGC GGC-3'; reverse, 5'-CCC ACT GCC TCC CGT AAG GAG T-3'). A Rotor-Gene 2000 Real-Time Cycler machine (Corbett Research, Australia) was employed in the generation of PCR products. Controls were prepared by omitting one of the primers and by analyzing RNA without reverse transcription. Data were analyzed using software provided by the manufacturer. The results shown are representative of three independent experiments.

Construction of a *P. chlororaphis* O6 *phzA*-Deficient Mutant

The *phzA* gene in the pPOE14 plasmid was disrupted by insertion of a 0.9-kb BamHI fragment, harboring a

kanamycin-resistance gene from the pRL648 plasmid [6], into a unique BamHI site within the open reading frames (ORF). The interrupted gene was exchanged into the chromosome, using previously described vectors [21]. The putative mutants were selected on the basis of kanamycin resistance and sensitivity to agar medium containing tetracycline and 5% sucrose. In order to verify the disruption, genomic Southern hybridization of DNA from the putative mutants was conducted with dig-labeled *phzA* PCR probes. To restore phenazine production to the *phzA*-deficient mutant, tri-parental mating was conducted using the mutant as the recipient, the pOphz1 cosmid clone containing the full-length *phz* biosynthetic operon as the donor, and pRK2073 as the helper strain. The transconjugants were selected on the basis of tetracycline resistance, and the restoration of phenazine production on KB medium via was determined by observation of orange coloration. The cosmids in these putative complemented transconjugants were isolated in order to verify the presence of the full *phz* operon within the plasmid.

Phenotypic Analysis of *gacS* or *phzA* Mutants

Phenazines were extracted from bacterial cultures with ethyl acetate and quantified via UV-visible light spectroscopy at 367 nm wavelength [18]. For quantitative analysis of hydrogen cyanide, cells were grown for 48 h in 50 ml of KB broth supplemented with 20 mM glycine, 20 mM $FeCl_3$, and appropriate antibiotics, in a 250-ml flask containing an inner well filled with 5 ml of sterile 1 M NaOH. HCN trapped by NaOH in the inner well was quantified by the method described by Lambert *et al.* [19], using hydantoin-pyridine and *N*-chlorosuccinimde-succinimide reagents, and HCN as a standard. The analyses were conducted in triplicate for each of the two trials. Culture cyanide levels were normalized at a culture optical density of 600 nm.

Effect of Bacterial Inoculations on Seed Germination

To assess the effects of wild-type *P. chlororaphis* O6, *phzA*- or *gacS*-deficient mutants and the complemented mutants on seed germination in wheat (cultivar Manning), barley (cultivar Saessal), cucumber (cultivar Baiksung 3), and rice (cultivar Dongjin) seeds were surface-sterilized for 10 min in 10% sodium hypochlorite and washed in 10 l of sterile distilled water. The seeds were immersed for 10 min either in suspensions of bacterial strains [10^{10} or 10^8 colony-forming units (CFU)/ml] or in sterile water, which was used as a control treatment. The seeds were thoroughly drained. In order to assess the CFU retained by a seed, four aliquots each of three seeds were transferred to 10 ml of sterile water and vortexed for 30 sec. Serial dilutions were plated on LB medium. The colonies were counted after three days of incubation at 28°C. The water- or bacteria-treated seeds were transferred to moist filter paper and incubated for three days in a growth chamber at 28°C. After the second day, sterile water was added to the paper on a daily basis. When the emerging shoots were equivalent in length to the seeds, the seeds were considered to have germinated. This experiment was repeated four times, using 50 seeds in each study.

The study was repeated to determine whether the metabolites responsible for seed germination inhibition were secreted. Thus, cells of O6 wild-type, the *gacS* and the *phzA* mutants, and their complemented mutants were grown in King's medium B for two days. Culture filtrates were prepared by centrifugation, followed by filtration with 0.2-μm membranes to remove residual cells. The surface-sterilized wheat seeds were immersed into the culture filtrates or noninoculated King's medium B. Germination was recorded after incubation in a growth chamber for three days at 28°C. Two-hundred seeds were used for each treatment, and the study was replicated three times.

Assessment of Phenazine Production from *P. chlororaphis* O6-Colonized Wheat Seedlings

Surface-sterilized wheat seeds were inoculated via immersion into a suspension of *P. chlororaphis* O6 (1×10^6 CFU/ml). The seeds were transferred for growth into sterilized, moistened vermiculite (300 ml Vermiculite:125 ml water). Other seeds were planted without bacterial inoculation for control treatment. After seven days of growth without additional watering, seedlings were removed, and seeds and roots were separated with a sterile blade. Seed and root segments were treated with ethanol (1 seed or 1 root/ml) and agitation for 5 min at 22°C, and the absorbance of these solutions was measured at 356 nm to determine the level of phenazines [22].

Assessment of Induced Systemic Resistance

Induced systemic resistance was determined using the methods previously described [26] with tobacco cv. Xanthi seeds grown on microtiter plates containing one-half strength Murashige and Skoog salt (MS) medium (GIBCO-BRL, Rockville, MD, U.S.A.), supplemented with 0.5% Phyta gel and 3% sucrose, and adjusted to pH of 5.7. Two weeks after seeding in microtiter plates, the roots were treated with either 10 μl of bacterial cultures (1×10^8 CFU/ml) or with sterile water as a negative control. One week after the bacterial treatments, the tobacco leaves were challenged with *E. carotovora* subsp. *carotovora* SCCI by pipetting 10 μl of inoculum onto the leaf. The *E. carotovora* cells were grown for two days on tryptic soy agar plates at room temperature, after which the cells were washed in sterile water and suspended in sterile distilled water to 1×10^8 CFU/ml. Two days after pathogenic challenge, disease was assessed by counting the number of leaves displaying soft rot. This experiment was repeated at least three times with at least 27 plants/treatment.

Data Analysis

Data were analyzed via ANOVA using SPSS 12.OK for Windows software (SPSS Institute, Republic of Korea). The significance of the effects of bacterial treatment was assessed by Duncan's multiple range test (p=0.05).

RESULTS

gacS Expression is Required for the Inhibition of Seed Germination by *P. chlororaphis* O6

The inoculation of wheat seeds with *P. chlororaphis* O6 impaired seed germination. With the "Manning" wheat cultivar, the germination of the control seeds was 80±3%, as compared with the 33±3% germination of seeds treated with a suspension of 10^8 CFU/ml *P. chlororaphis* O6. Diffusion of orange-colored phenazines into filter papers around the inoculated seeds was observed. Treatment of wheat seed with inoculum concentrations of greater than 10^6 CFU per seed was required to inhibit the germination, and less than 10^5 CFU concentrations per seed did not result in inhibition (data not shown).

When the roots of one-week-old barley or wheat roots were inoculated with *P. chlororaphis* O6 via seed coating, no inhibitory effects on root development were observed (data not shown). In order to gain further insight into the relationship between phenazine formation and root length, the germinating seeds and roots were separated from wheat seedlings grown from *P. chlororaphis* O6-inoculated seeds. The data shown in Table 1 indicate that phenazines were more abundant in the seed area than in the root area.

The inhibitory effects of *P. chlororaphis* O6 on seed germination were plant host-dependent. For barley and wheat seeds, approximately 80% reduction of germination was observed with *P. chlororaphis* O6 inoculation, but no inhibition was observed with cucumber or rice seed inoculations (Fig. 1). Furthermore, no inhibition of germination was observed with wheat, barley, rice, or cucumber when the seeds were treated with cells of the *gacS* mutant (Fig. 1). For wheat and barley, the inhibition of seed germination was restored *via* complementation of *gacS* mutant with the wild-type *gacS* gene (Fig. 1).

Table 1. Production of phenazines in wheat seedlings colonized with *P. chlororaphis* O6.

Sample	Absorbance (356 nm) of the ethanol fraction[a]	Mass (g)
Roots	0.11±0.11	1.50±0.50
Seed	2.50±0.50	1.13±0.05

[a]The average mass of plant material extracted is shown in grams. Data are means of two separate experiments, each with five plants, and standard errors.

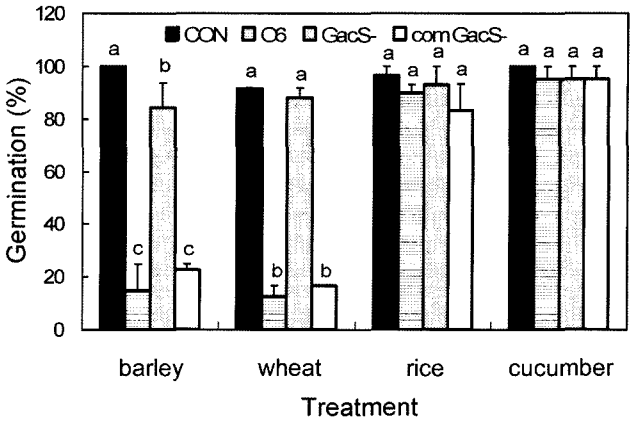

Fig. 1. Effects of *Pseudomonas chlororaphis* O6 (O6), its *gacS* mutant (GacS$^-$), and the complemented mutant (com GacS$^-$) on seed germination.

The inoculum was applied at 1×10^9 CFU/seed, and control (Con) seeds were treated with water. Percent germinations were scored seven days after bacterial treatment. The data shown are expressed as means of three independent experiments, each using 50 seeds per treatment, and the vertical bars indicate standard errors. Different letters indicate significant differences between treatments, according to the results of Duncan's multiple range test at p=0.05.

Regulation of Expression from *phzI* and *phzA* by GacS

Sequence analysis of the *P. chlororaphis* O6 *phz* operon indicated the presence of *phzI* and *phzR* regulatory gene homologs, a biosynthetic operon with potential *phzABCDEFG* genes, and a *phzO* gene, which encodes for an enzyme converting phenazine-1-carboxylic acid to 2-OH-phenazine carboxylic acid in *P. chlororaphis* O6 (data not shown). The potential PHZ-box palindromic sequences, ACTACAAGA/TCTGGTAGT for the *phzA* gene and CCTACCAGA/TCTTGCAGG for the *phzI* gene, were located upstream of the putative *phzI* and *phzA* genes (data not shown). These functional genes had 90% similarity with those of other pseudomonads.

Transcript levels of the *phzA* and *phzI* genes in the wild-type cells increased in abundance with the age of culture (Fig. 2A); however, the fold change (maximally about 2 fold) was not as great as that observed for the *gacS* transcripts (Fig. 2B). A mutation in *gacS* resulted in reduced accumulation of *phzA* and *phzI* transcripts (Figs. 2C and 2D) with the effect being greater for *phzA*. In the complemented *gacS* mutant, production of the *phzI* transcript was significantly increased over the level observed in the wild-type (Figs. 2C and 2D).

Effect of a *phzA* Mutant on the Inhibition of Seed Germination and the Induction of Systemic Resistance

The mutation in *phzA* eliminated the production of orange-colored phenazines for the cells cultured in KB medium, but HCN generation was not impaired (Table 2). The inoculation of seeds with this phenazine-deficient, HCN-

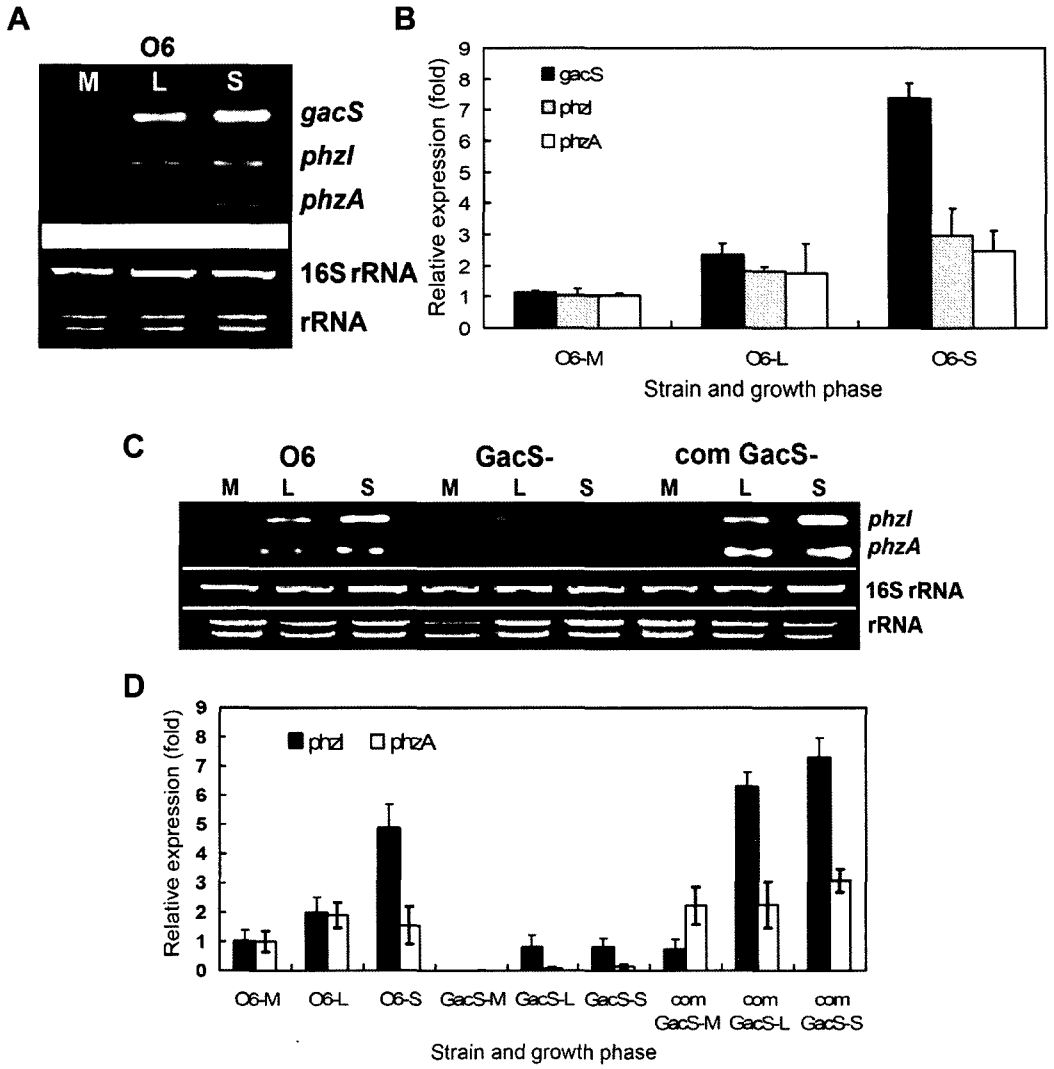

Fig. 2. Effects of growth phase on transcript abundance from *phzI*, *phzA*, and *gacS* in *P. chlororaphis* O6 wild-type (O6, in **A**, **B**), the *gacS* mutant (GacS$^-$, in **C**, **D**), and the complemented *gacS* mutant (com GacS$^-$, in **C**, **D**).
RNA was extracted from the KB-grown cultures at different stages of growth (M, mid-log phase cells; L, late-log phase cells; S, stationary phase cells). Transcript levels were compared with transcript levels from the mid-log phase cells of the wild-type *P. chlororaphis* O6, which were set at 1. Data shown represent one of three studies that yielded similar results.

Table 2. Production of hydrogen cyanide from cell suspensions of wild-type and mutant strains of *P. chlororaphis* O6.

Strain	Cyanide production
	Concentration in culture fluid (μmol per culture OD 600 nm)[a]
Wild-type	1,081±615
gacS mutant	15±7
Complemented mutant	879±837
Wild-type	435±241
phzA mutant	521±445

[a]Data are means of two separate trials totaling 6 replicates±standard deviation. Values (absorbance of 600 nm) from control broths were 3±2 μmol per culture (N=7).

generating *P. chlororaphis* O6 mutant caused less inhibition of germination in barley and wheat seeds than those treated with the wild-type strain (Fig. 3). The treatment of barley and wheat seeds with wild-type *P. chlororaphis* O6 cells resulted in only 10–20% germination, as compared with 60–70% seed germination observed when *phzA* mutant cells were used. Inhibition of germination was increased to a higher level by *phzA* complementation (Fig. 3). Germination of rice and cucumber seeds was not affected by inoculation with the *phzA* or the complemented *phzA* mutant (Fig. 3). Treatment of wheat seeds with culture filtrates from *phzA* and *gacS* mutants did not inhibit germination, whereas germination of seeds was inhibited with culture filtrates from wild-type and complemented mutant strains (Fig. 4).

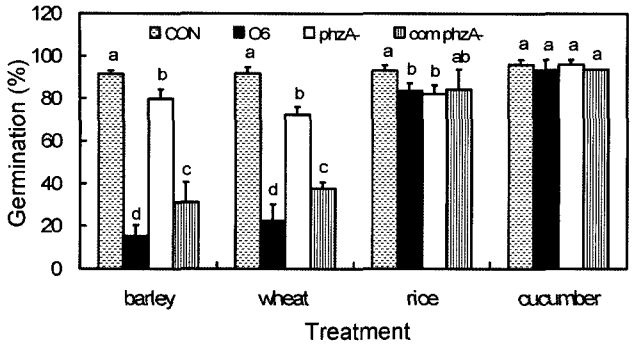

Fig. 3. Germination of seeds treated with the wild-type strain (O6), the phenazine-deficient *phzA* mutant (phzA$^-$), and the complemented mutant (com phzA$^-$) of *P. chlororaphis* O6.

The seeds were inoculated at 1×10^{10} CFU/ml. Germination was scored seven days after bacterial treatment. The data shown are expressed as means of three studies each, using 50 seeds per treatment, and the vertical bars indicate standard errors. Different letters indicate significant differences between treatments, according to the results of Duncan's multiple range test at p=0.05.

Although root colonization by wild-type *P. chlororaphis* O6 conferred systemic resistance against the soft-rot pathogen in tobacco, protection of plants with roots colonized by the *phzA* mutant was reduced (Fig. 5); the disease rating was between 10–13% in plants colonized by the wild-type strain, whereas it was 40–45% in tobacco colonized by the *phzA* mutant. Disease in the control plants lacking inoculated root systems was rated as 60–70%. Complementation of *phzA* mutation restored protection to the level achieved in plants colonized by the wild-type strain.

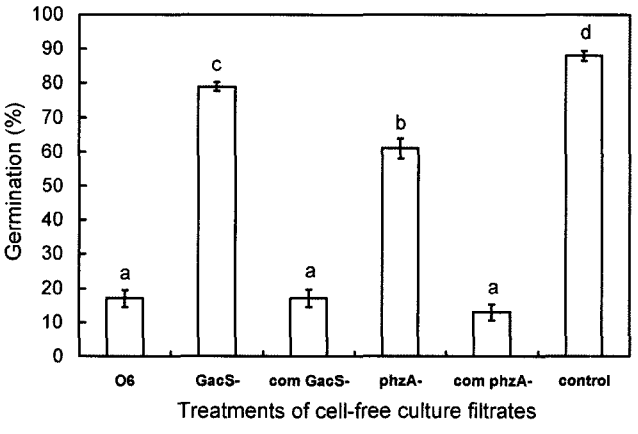

Fig. 4. Effects of cell-free culture filtrates of *Pseudomonas chlororaphis* O6 (O6), the *gacS* mutant (GacS$^-$), the complemented mutant (com GacS$^-$), the *phzA* mutant (phzA$^-$), the complemented *phzA* mutant (com phzA$^-$), and the sterile growth medium (Con) on seed germination of wheat.

The data shown are expressed as means of three independent experiments, each using 200 seeds per treatment, and the vertical bars indicate standard errors. Different letters indicate significant differences between treatments, according to the results of Duncan's multiple range test at p=0.05.

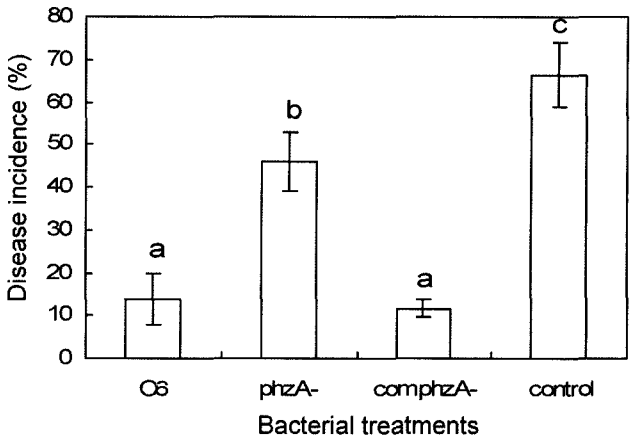

Fig. 5. Induced systemic resistance against a soft-rot pathogen, *E. carotovora* SCC1, in tobacco seedlings with roots colonized by *P. chlororaphis* O6 (O6), the *phzA* mutant (phzA$^-$), or its complemented mutant (com phzA$^-$), or treated with water as a control.

Data are expressed as means of three independent experiments, with 21 plants per treatment. Different letters indicate significant differences in the incidence of soft-rot symptoms between the treated and control samples, on the basis of Duncan's multiple range tests at p=0.05.

DISCUSSION

Inhibition of wheat and barley seed germination required seed coating by *P. chlororaphis* O6 above the level likely to occur from inoculation with bacteria present in field soil but at levels that could be reached by commercial seed coating. Our findings confirmed the inhibitory effect of wheat seed coating on germination by *P. fluorescens* 2-79, which produces phenazines with structures different to *P. chlororaphis* O6 [25].

Although inhibition of seed germination is correlated with HCN production by other rhizobacteria [3, 8, 17, 24], phenazines rather than cyanide production have been suggested to be the major contributor to inhibition of germination of wheat and barely seeds by *P. chlororaphis* O6. This concept was confirmed in the present study by assaying seed germination with culture filtrates: inhibition by the wild-type and complemented *gacS* and *phzA* mutants, but not by *phzA* or *gacS* mutants, correlated with the secretion of phenazines when the wild-type genes were present. Although still able to produce cyanide, the *phzA* mutant did not inhibit wheat and barley seed germination to the same extent as the wild-type strain. Loss of phenazine production in the *gacS* mutant was also consistent with its lack of inhibitory effect on seed germination. Phenazines are known to alter redox balance in cells [4, 10], and such an alteration may occur in plant cell metabolism to impair seed germination, when *P. chlororaphis* O6 is present on the seed.

Rice and cucumber showed no inhibition of seed germination by *P. chlororaphis* O6. It is possible that differences in the composition of seed exudates caused such

differential effects. Indeed, although orange colored phenazines were visually produced when inoculated seeds of wheat and barley were incubated, no such coloration was observed with the inoculated seeds of rice and cucumber. AHSL and phenazine production from *P. chlororaphis* O6 were reduced in a minimal medium containing defined carbon and inorganic nitrogen sources, as compared with the nutrient-rich KB medium (unpublished data). The presence of shikimic acid and phenylalanine in medium has been shown to enhance *phz* expression in another phenazine-producing pseudomonad [7]. Thus, differences in the production of such inducing compounds from different seeds could influence the extent to which phenazines are produced.

Another role of the production of phenazines is to induce systemic resistance against the pathogen *E. carotovora* that causes soft-rot disease. Previously the phenazine, pyoryanin, produced by (*P. aeruginosa*) was correlated with induced resistance against *Botrytis cinerea* in the tomato [1]. These findings partly explain the reason why the *gacS* mutant of *P. chlororaphis* O6, which was deficient in phenazine production, did not induce systemic resistance to the soft-rot pathogen [26]. The fact that the *phzA* mutant still induced some level of resistance might be attributed to the production of additional stimulants, such as butanediol: *P. chlororaphis* O6 produces butanediol, a stimulant of growth and systemic resistance under *gacS* control [11].

Our analysis of transcript abundance led us to deduce that GacS is a prerequisite for *phzA* expression, similar to other pseudomonads [9, 27]; however, other control mechanisms may exist for *phzI*. Low transcript levels were detected in the *gacS* mutant in the late-log and stationary phases. Additionally, the complementation of the *gacS* mutant resulted in higher accumulations of transcripts for *phzI* than observed with the wild-type strain.

These studies underline the complexity and differential nature of interactions between metabolites of rhizosphere bacteria and their host plants. Phenazine production by *P. chlororaphis* O6 is a beneficial trait; it directly inhibits fungal pathogen growth and induces systemic resistance. Our finding that phenazine levels were higher in the seed zone than on the surfaces of developing roots of seedlings growing from *P. chlororaphis* O6-inoculated seeds suggests that direct inhibition of pathogen growth is likely to be more effective near seeds rather than roots. However, phenazine production by root colonizers also has a negative effect through inhibition of seeds germination of some plants such as wheat and barley. Thus, the dose of bacterial inoculant may have to be considered in commercial seeds.

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